STUDIES ON SYNTHETIC AND NATURALLY OCCURRING GLYCOSIDASE INHIBITORS FROM MUSHROOMS

by
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A thesis submitted in partial fulfilment of the requirements for the degree of Master of Philosophy

June, 1994
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Acknowledgements

I would like to express sincere thanks to my supervisors, Dr. Yum-Shing Wong and Dr. Paul Pui-Hay But for their tireless guidance during the period of study and valuable assistance in editing and proofreading the manuscript. Special thanks go to Dr. Tony K. M. Shing in the Department of Chemistry. He kindly allowed me to work in his laboratory. His friendship and suggestions are much appreciated. For Dr. P. K. Wong and Prof. J. C. Lagarias, I thank them for their willingness to serve on my thesis committee.

I was also greatly indebted to Mr. Vincent W.-F. Tai and Mr. L.-H. Wan for their assistance in purification of compounds. Preparation of reference standard (arabitol) and recording NMR spectra was also kindly done by Mr. Vincent W.-F. Tai. Thanks are also given to the Shanghai Institute of Organic Chemistry for mass spectra analysis. I also wish to appreciate the graduate students in Room G92 and G53 Science Centre and technical staff especially Mr. Thomas C.-O. Tang for their technical assistance, encouragement and helpful discussions.

Finally, I acknowledge once again the help that many individuals have given me during the past two years.

This research project was financially supported by the Research Grants Council (to Dr. Y.-S. Wong) and the Croucher Foundation (to Dr. T.K.M. Shing).

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Abstract

Glycosidase is a group of hydrolases which attack the glycosidic bond with the terminal release of sugar. They are enzymes involved in carbohydrate metabolism and glycoprotein processing. Inhibition of these enzymes has implication for anti-viral chemotherapy by alteration of the glycoprotein coat of virus.

A novel pseudo-sugar, cyclophellitol was first isolated from the fungus, *Phellinus* sp., in 1989. It has cyclitol structure with ß-epoxide and inhibited the almond ß-D-glucosidase with low IC_{50} value, 0.8 μg/ml.

In this study, the glycosidase inhibitors were isolated from *Ganoderma lucidum* which belongs to the same family as *Phellinus*. Aqueous extract of *G. lucidum* was shown to have potent inhibitory effects on brewers yeast α-D-glucosidase and *E. coli* α-D-galactosidase. It also slightly inhibited almonds ß-D-glucosidase. Two compounds with strong inhibitory effects on α-D-glucosidase were purified from aqueous extract of *G. lucidum* by liquid chromatography. One was identified as an equilibrium mixture of D-glucose. The other compound was characterized as arabitol by mass spectrophotometry and nuclear magnetic resonance.

In addition, two classes of synthetic glycosidase inhibitors were prepared by the Chemistry Department of CUHK. Each compound was screened for its inhibitory effect on six glycosidases namely, α- and ß-D-

glucosidases, α- and ß-D-mannosidases and α- and ß-D-galactosidases, in order to correlate the structure-function relationship.

One series of synthetic glycosidase inhibitors studied are cyclophellitol and its analogues which have similar epoxide ring in the molecule. All compounds were found to inhibit configurationally related glycosidase specifically. They inhibited the enzyme by forming irreversible enzyme-inactivator complex. These inhibitors are also named as inactivators. Another series of synthetic compounds studied are aminocyclitols. They are potential reversible inhibitors which exhibited a competitive mode of inhibition. By means of structure-function studies of all these compounds, the important requirements for these potential inhibitors to exhibit inhibitory activities on glycosidases could be revealed.

Chapter I: Introduction

Glycosidase is group of hydrolases which attack the glycosidic bond of carbohydrates, glycoproteins and glycolipids with the terminal release of monosaccharide unit or sugar chain.[8] They are enzymes involved in carbohydrate metabolism and glycoprotein processing. Inhibition of these enzymes has implication for anti-viral chemotherapy by alteration of the glycoprotein coat of virus.

Some known glycosidase inhibitors such as deoxynojirimycin and castanospermine isolated from plants are sugar analogues.[15-17,38,41] They inhibit the glycosidases due to their structure bearing a greater resemblance to the natural substrate and competing for the active site of the enzyme. In 1989, a novel pseudo-sugar cyclophellitol was isolated from the fungus, *Phellinus* sp. It was characterized as an effective ß-D-glucosidase inhibitor with low IC_{50} value, 0.8 μg/ml.[2-3,43,50,52-54,59]

In this study, one objective is to search for naturally occurring glycosidase inhibitors from *Ganoderma lucidum*, a mushroom of the same family as *Phellinus* and known to have medicinal value.(Chapter IV)

Two classes of pseudo-sugars synthesized by the Chemistry Department of CUHK were studied to characterize their effects on glycosidase inhibition (Chapter III). One series of synthetic inhibitors is cyclophellitol and its analogues which have similar epoxide ring in the molecule. These compounds inhibit the enzyme by forming irreversible enzyme-inhibitor complex. They

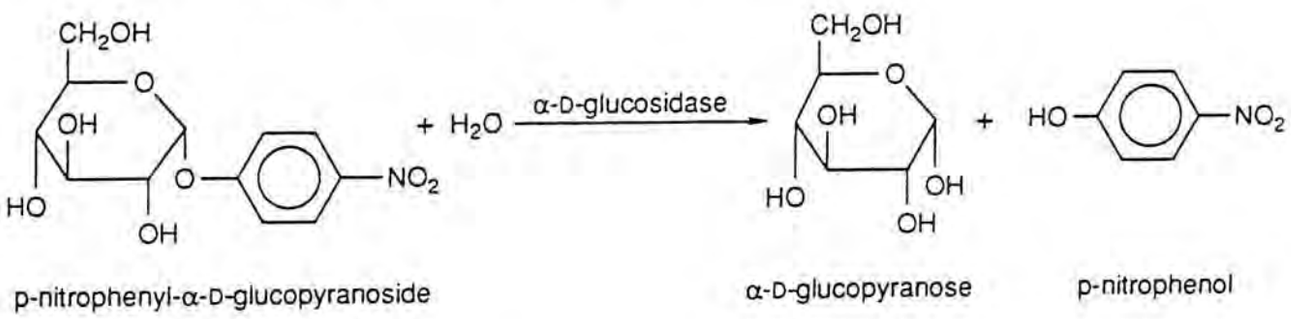
are also named as inactivators.[59] Another series of synthetic compounds are aminocyclitols. They are the potential reversible competitive inhibitors. All compounds were screened for their inhibitory effects on six glycosidases namely, α- and ß-D-glucosidase, α- and ß-D-mannosidases, and α- and ß-D-galactosidases in order to correlate their structure-function relationship. Such attempts could give information on the specificity of the inhibitor on its target enzyme.

Chapter II: Literature Reviews

II.1 Glycosidases

Glycosidases (EC 3.2) are classified under hydrolases, although some of them can also transfer glycosyl residues to oligosaccharides, polysaccharides and other alcoholic acceptors. These enzymes catalyse the hydrolytic cleavage of glycosidic bond in carbohydrate, glycoproteins and glycolipids with the terminal release of a monosaccharide or sugar chain. They show specificity to *O*-, *N*- or *S*-glycosidic bonds and are subdivided into three classes responsible for hydrolysing *O*-glycosyl, *N*-glycosyl and *S*-glycosyl compounds (EC 3.2.1, EC 3.2.2, EC 3.2.3 respectively).[8] In this study, glycosidase hydrolysing *O*-glycosyl compounds(EC 3.2.1) including α- and ß-D-glucosidases, α- and ß-D-mannosidases, and α- and ß-D-galactosidases were employed. Artificial substrates were used in enzyme assays. These enzymes were chosen because the most common carbohydrates found in natural glycoprotein molecules are glucose, mannose and galactose, respectively.

The general catalytic reaction is as follows:



p-nitrophenyl-α-D-glucopyranoside α-D-glucopyranose p-nitrophenol

The product, p-nitrophenol, can be monitored colorimetrically.

II.2 Biosynthesis of N-linked Glycoprotein[13,29]

Glycosidases are key enzymes in the biosynthesis of N-linked glycoproteins. They play a critical role in the trimming of glycosyl side chains to produce hybrid and complex types of N-linked oligosaccharide units (see Fig. II.1). All N-linked oligosaccharides are assembled in the endoplasmic reticulum by the stepwise addition of various sugars to the lipid carrier dolichol-phosphate to form the oligosaccharide donor, $Glc_3Man_9(GlcNAc)_2$-pyrophosphoryl-dolichol. This oligosaccharide donor is then transferred to specific asparagine residues of the polypeptide chain. Once the oligosaccharide has been transferred to protein, it undergoes processing reaction that results in the removal of some sugars and the addition of others. The initial reactions, which have been shown to occur in the endoplasmic reticulum, involve the removal of all three glucose residues by two membrane-bound α-glucosidases (Glucosidase I and II[34]). The removal of all 4 α1,2-linked mannoses by mannosidase I then follows. A GlcNAc transferase (GlcNAc TI) subsequently adds a GlcNAc to the 3-linked mannose followed by removal of the α 1,3 and α 1,6-linked mannoses by mannosidase II. The resulting product can then be further glycosylated by addition of GlcNAc, galactose, sialic acid, and fucose to give a wide variety of complex chains.[13]

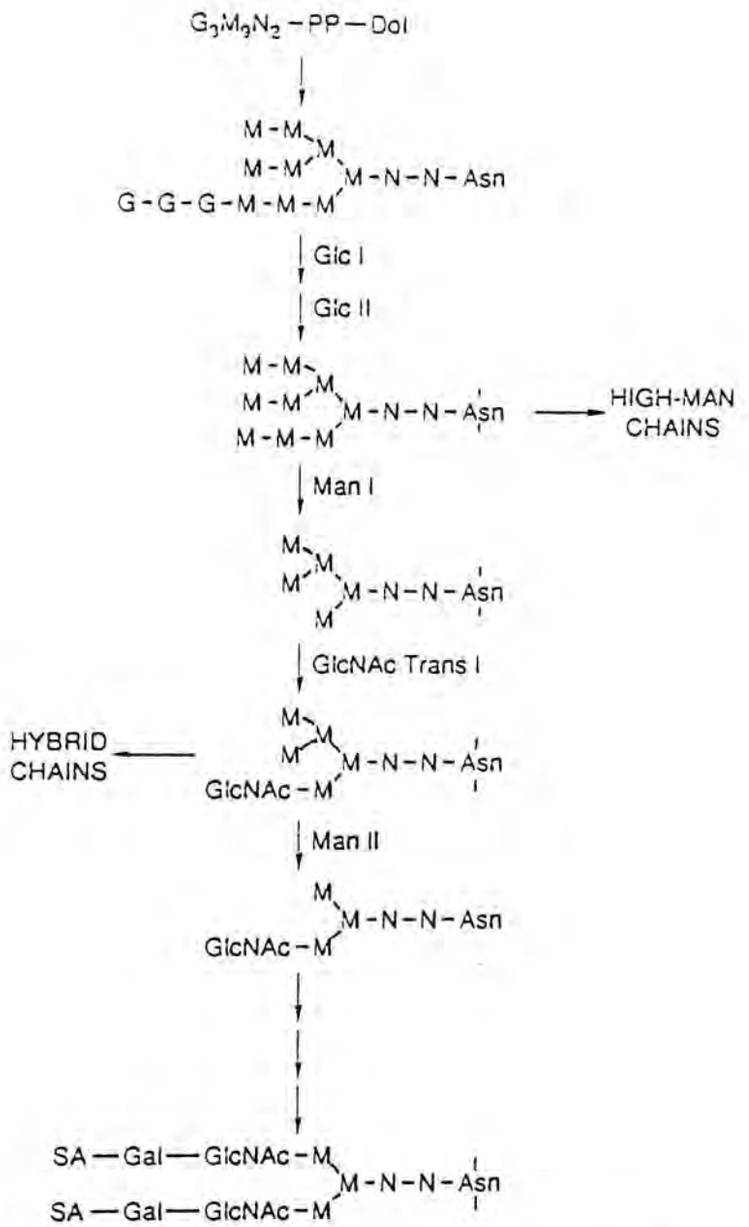


Fig. II.1 The reactions involved in the processing of the $Glc_3Man_9(GlcNAc)_2$ structure giving rise to various types of high mannose, hybrid, and complex types of oligosaccharides. G = glucose, M = mannose, N = GlcNac(N-acetylglucosamine), Glc = glucosidase, Man = mannosidase, SA = sialic acid, Gal = galactose, Asn = asparagine.[13]

When the glycosidase inhibitors were administered in the animal cell in culture, it was revealed that the oligosaccharides in N-linked glycoprotein were altered. Such alteration would in turn affect the cell function.[13,27,42]

As glycosidases are key enzymes in the biosynthesis of N-linked glycoprotein, inhibition of these enzymes have aroused the interest of anti-viral chemotherapy by affecting the proper maturation of viral envelope glycoprotein. Some glucosidase inhibitors, such as castanospermine and 1-deoxynojirimycin, have been reported to inhibit syncytium formation and infection of human immunodeficiency virus (HIV).[21,57] HIV, the causative agent of AIDS, is tropic for cell displaying the CD4 surface protein, with the resulting formation of multinucleated syncytia and cell death. Interaction of HIV with cell is mainly dependent on a binding reaction of glycoprotein 120 (gp120) of the virus and CD4 molecules of the cell. It involves N-linked glycan. Both gp120 and CD4 are heavily glycosylation. Interference of the glycosylation step of the above glycoprotein would affect cell fusion and inhibit the formation of syncytia. The cytotoxic consequence of HIV infection would greatly decrease after treatment by inhibitor. The glycosidase inhibitors may offer realistic prospect of a new therapy for treating HIV infection in the future based on these exciting evidences. The above two compounds have also been reported to inhibit leukaemia virus at non-toxic concentration and herpes virus cytomegalo virus.[17]

Glycosidases also play a vital role in carbohydrate catabolism and turnover of many cellular components such as polysaccharides, glycolipids and proteoglycans. Inhibition of these enzymes could also have promising therapeutic application. For example, they could be useful as antihyperglycemic agents for treatment of diabetes. Deoxynojirimycin and several semi-synthetic derivatives were found to inhibit the rise in blood glucose level after meal. They acted as anti-diabetic drugs by inhibiting the digestive glycosidase. They are also treated as inhibitors of tumor metastasis by changing the saccharide structure on tumor cell surface, as drugs to prevent obesity, as antifungal agents or as agents to interfere with insect feeding. One possibility of glycosidase inhibitors as insect feeding deterrence is that they compete with sugar for a binding site on the sugar-sensitive neuron in taste sensilla without triggering a response. Alternatively, it has been suggested that a glucoside is present at or near the sugar receptor site and plays a part in its function. The presence of these compounds in plants and fungi may render insects temporarily unable to detect sugars and is associated with reduced feeding.[17]

Most of the above mentioned effects are resulted from direct or indirect inhibition of glycosidase enzymes.

II.3 Mechanism of Enzyme-Catalysed Reaction

In order to study the glycosidase inhibitors, a thorough understanding of the catalytic mechanism of the enzyme is necessary. Glycosidase-catalysed hydrolysis actually resembles the non-enzymatic acid-catalysed hydrolysis of simple alkyl or arylglycosides in that cleavage of the glycosyl (C_1-oxygen) bond occurs.[5,33] This has so far been demonstrated with ß-amylase of barley and sweet potato, α-amylase of hog pancreas and *B. subtilis*, glucamylase from *A. niger*, α-glucosidase of brewers yeast, ß-glucosidase of almond emulsin, lysozyme, ß-galactosidase of *E. coli*, and ß-glucuronidase from calf liver.[5]

In non-enzymatic hydrolysis of glycosides, an SN1-like reaction (nucleophilic, first order) is favourable because the glycosyl cation intermediate can be stabilized by charge distribution between C-1 and the ring oxygen atom (see Fig.II.2). In contrast, an SN2 (nucleophilic, second order) mechanism is unfavourable and therefore much slower. The direct displacement of the aglycon by hydroxide or a water molecule is strongly hindered due to the inversion at the anomeric carbon atom. This would result in an intermediate of the sugar having unfavourable skew or boat conformation, making the activation energy prohibitively high.[32]

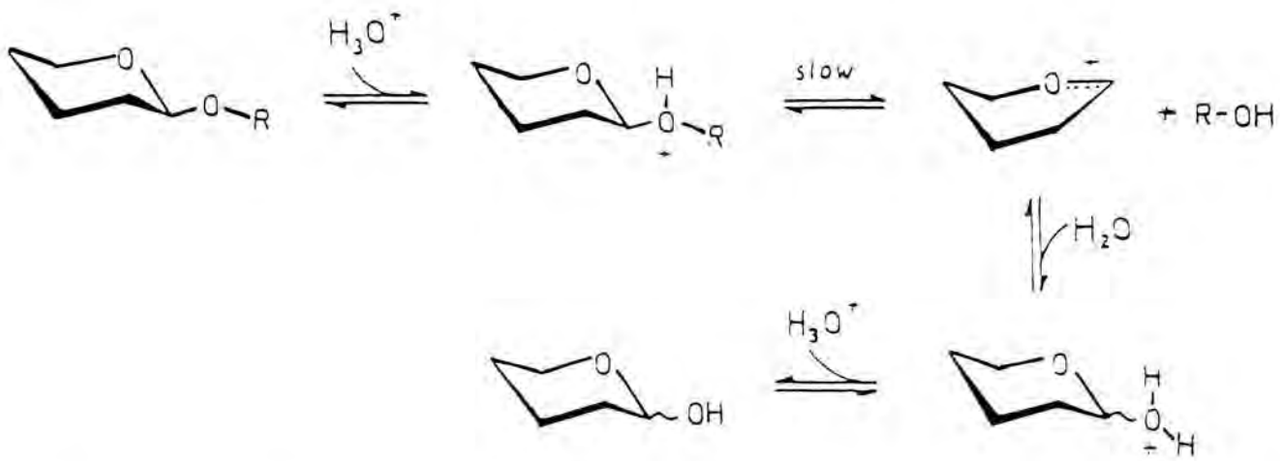


Fig. II.2 Acid-catalysed hydrolysis of ß-glycoside (hydroxy substituents omitted).[32]

The enzyme catalysed reaction occurs much faster by lowering the activation energy barrier. It achieves this in the following ways. First, intrinsic weak bonds ensure the enzyme-substrate and enzyme-product complexes to form and dissociate rapidly. Other factors which lower the activation barrier include the conversion of a bimolecular reaction between the catalyst and substrate into a unimolecular one within the enzyme-substrate complex, the precise orientation of catalytic groups with respect to the bonds to be broken, multifunctional catalysis by two or more of such groups, formation of reaction intermediate, favourable changes of the local dielectric constant by the exclusion of solvent water, stabilization the transition state and destabilization the ground state relative to the conditions of the unbound, fully solvated substrate, all of these cause a rate acceleration corresponding to the decrease in the free energy of activation.[32]

With regard to enzyme-catalysed reactions, they are formally nucleophilic substitution at C-1 of the glycosides, and glycosidases may be divided conveniently into two classes according to whether the hydrolysis (or transfer) reactions which they catalyse proceed with inversion or retention of configuration.[5] Most mechanistic data come from enzymes that catalyse the reaction with retention of the configuration. They operate by double displacement mechanism (Fig. II.3). The first step is always considered to be the protonation of the anomeric oxygen by an AH group of the enzyme which leads to the release of the aglycon moiety. The product is a covalent glycosyl enzyme intermediate ES of inverted configuration which is formed through the oxocarbonium ion-like transition state. The second step is decomposition of ES. The A^- group of the enzyme is involved as a general base catalyst. The enzyme-catalysed reaction is completed by the stereospecific addition of a hydroxyl group to the oxocarbonium ion. Water or another hydroxylic compound acts as a hydroxyl donor in hydrolysis. The product yielded has the same configuration as the starting glycoside with the retention of configuration.[5,33]

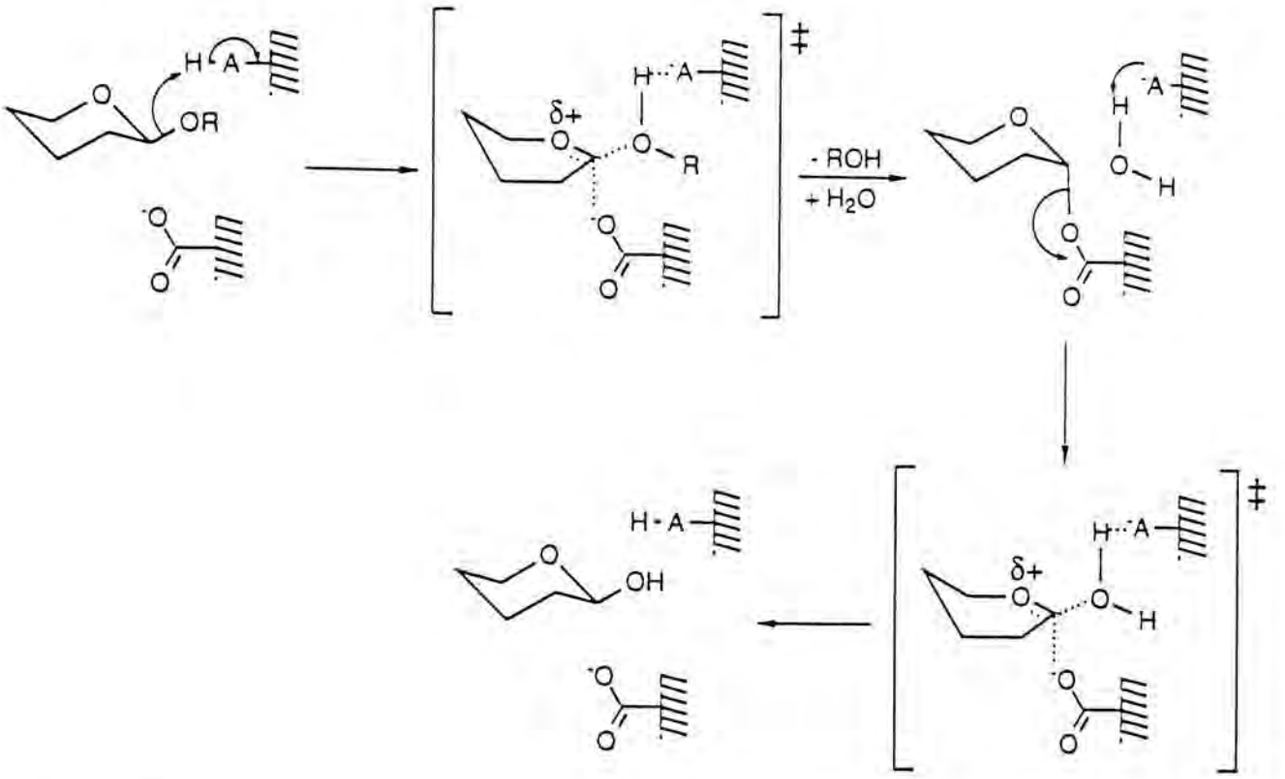


Fig. II.3 A general pathway of the double displacement mechanism for retaining glycosidases. It was first proposed by Koshland and recently reviewed by Sinnot.[7]

In lysozyme, it was proposed that they are Glu-35 acting as a general acid catalyst (AH) to protonate the glycosyl oxygen atom, and carboxylate of Asp-52 stabilizing the carbocation formed. Carboxylate of aspartate acts as nucleophile has also been shown in the case of ß-glucosidase A3 of *Aspergillus wentii* and that of almonds but in *lacZ* ß-galactosidase of *Escherichia coli*, it is Glu-461.[14,45]

II.4 Types of Glycosidase Inhibitors

A great variety of naturally occurring and synthetic compounds have the ability to bind reversibly or irreversibly to specific enzymes and alter their activities. They are named as enzyme inhibitors by reducing or eliminating the catalytic activity of the enzyme. Two general classes of inhibitors are recognised according to whether the inhibitory action is irreversible or reversible. In irreversible inhibition, the inhibitor becomes covalently linked to the specific functional group, usually an amino acid side chain which is associated with the catalytic activity. The dissociation of the inhibitor from the enzyme is therefore cannot be released by dilution or dialysis. The velocity of the reaction is reduced to an extent that corresponds to the fraction of enzyme molecules that have been inactivated. In contrast, reversible inhibition is characterized by transient association of inhibitors with the enzymes. Three distinct types of reversible inhibition are known. Competitive inhibitors resemble the substrate and compete with substrate binding to the active site of the enzyme. They diminish the rate of catalysis by reducing the proportion of enzyme molecules that have bound substrate. Increased Km value but no change in Vmax is obtained in kinetic expressions for conversion of substrate to product. In noncompetitive inhibition, both the inhibitor and substrate bind simultaneously to an enzyme molecule. They act by decreasing the turnover number of the enzyme molecule. Turnover number represents the number of moles of substrate that one mole of enzyme is able to transform per minute.

Km is not affected by this kind of inhibition but Vmax will decrease. It is kinetically distinguishable from the competitive type of inhibition. The inhibitors which can bind reversibly with the enzyme-substrate complex but not the free enzyme are designated as uncompetitive inhibitors. Both Km and Vmax are decreased.[9]

In fact, enzyme inhibitions are not limited to these mechanisms. Other inhibition such as mixed type inhibition, substrate inhibition and allosteric inhibition will not be discussed in this thesis.

With regard to glycosidase inhibitors, these can be grouped into two general types. They are noncovalent/reversible competitive inhibitors and covalent/irreversible inhibitors (active site-directed). Noncovalent inhibitors include cationic and basic glycosyl derivatives, glycals, glyconolactones and thioglycosides.[33] Cationic and basic glycosyl derivatives are usually more potent than their neutral counterparts; for example ß-glucosylamine and its N-substituted products are bound 220 to 4000 times more tightly than ß-aldose on ß-glucosidase.[33] This is because the protonated intermediate of this compound can be stabilized by the negatively charged group at the active site in the enzyme. The correct position of cation or basic group is important for efficient interaction with the active site of target enzyme. The abundant alkaloidal glycosidase inhibitors (AGIs) are also considered as this type. AGIs occur in many species of higher plants, bacteria and fungi. They belong to five different chemical structural types, namely polyhydroxylated derivatives

of piperidine, pyrrolidine, pyrroline, octahydroindolizine, and pyrrolizidine. Examples of these are some well known compounds such as deoxynojirimycin and castanospermine. Some of the simpler piperidine and pyrrolidine derivatives bear an obvious structural resemblance to 1-deoxy monosaccharides, with the ring oxygen replaced by nitrogen. The orientation of hydroxy groups on the more complex bicyclic structures also suggests a structural resemblance to sugars.[17]

Glycals are a kind of pseudosubstrate having moderate inhibition on glycosidase. D-glycal itself is a weak inhibitor. The inhibition might be resulted from the slow formation of EI intermediate combined with slow hydrolysis later to regenerate the free enzyme.[32]

The strong inhibition of glycosidase by aldonolactones was first reported in 1940 by Japanese workers who studied ß-D-glucosidases from *Aspergillus* and almonds. These studies showed that aldonic acids themselves are non-inhibitors, and that 1,5-lactones are potent inhibitors. It was proposed that aldolactones exerting their powerful inhibition by virtue of their close similarity of conformation to that of the oxo-carbonium ion like transition state. Both the lactone and oxocarbonium ion have trigonal, planar configurations at C-1 position and adopt a half chair conformation.[32,33]

Thio-glycosides are compounds in which the sulphur replace the glycosidic oxygen. The rate of acid-catalysed hydrolysis was however decreased when using thio-glycosides derivatives as a substrate. Thus, they

are strictly competitive inhibitors.[33]

Covalent/irreversible glycosidase inhibitors inactivate the glycosidase by forming covalent bonds with the active site of the enzyme. There are several common structural requirements. First, the glyconic part of these compounds must provide the binding and the orientation in the enzyme active site. Second, the reactive group must be directly connected with the C-1 atom of the glyconic residue so that this group lies close to the catalytic groups within the enzyme-inhibitor complex. Thus, the covalent bond formed should be sufficiently stable.[44] Inhibitors satisfying these requirements include epoxide derivatives, isothiocyanate derivatives, N-bromoacetyl glycosylamine derivatives and triazine derivatives.[33] Epoxide derivatives are highly specific irreversible inhibitor of glycosidases.

The reaction involved is a general acid-catalysed ring opening of the epoxide by the carboxylic acid catalytic group of the enzyme which lies in close proximity. Then the nucleophilic carboxylate attackes this activated species leading to formation of a covalent ester bond.[32,44]

Isothiocyanate and N-bromoacetyl glycosylamines derivatives are both N-glycosides. ß-D-glucopyranosyl isothiocyanate and N-bromoacetyl-ß-D-galactosylamine are potent inhibitors of almonds ß-glucosidase and *E.coli* ß-galactosidase, respectively.[33,37,44] The former is an electrophile which readily reacts with amino groups near the active site of the enzyme at neutral pH to form thiourea derivatives. This property makes these class of compounds as

an active site directed inhibitor.[32] The N-bromoacetyl-ß-D-galactosylamine is a compound that alkylates a single methionyl residue near the active site of the enzyme.[37]

Several glycosidases have been studied with triazene derivatives of the corresponding glycosides. These inhibitors act with similar efficiency on the ß-galactosidase from human fibroblast lysosomes and the ß-xylosidase from *Penicillium wortmannii* but with much lower efficiency on ß-glucosidase from almonds and α-glucosidases from yeast and no effect on all other enzymes.[33] The inactivation mechanism involves alkylation of an amino acid at the active site.

Sugar-related aziridines are a newly developed type of mechanism-based enzyme inactivator.[6] The mechanism is assumed to be esterification of carboxylate group at the active site, a mechanism which is quite similar to epoxide derivatives mentioned before.[32]

These active-site directed irreversible inhibitors are useful to label the functional amino acid groups in the active site of an enzyme.

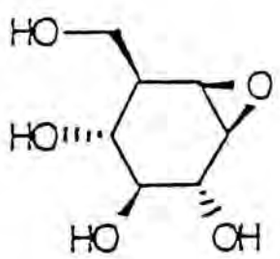
II.5 Cyclophellitol and Aminocyclitols

The pseudo-sugars studied and described in this thesis were synthesized by the Chemistry Department. They are diastereoisomers of cyclophellitol and valiolamine respectively. The mode of inhibition of the diastereoismers is believed to be similar to their parent compound. The following reviews provide the general backgrounds and references for testing these synthetic pseudo-sugars on glycosidase as described in Chapter III.

II.5.1 General background on cyclophellitol

Cyclophellitol is a novel ß-D-glucosidase inhibitor isolated from the culture filtrate of the fungus, *Phellinus* sp. The molecular formula is $C_7H_{12}O_5$, (*1S,2R,3S,4R,5R,6R*)-5-hydroxymethyl-7-oxabicyclo[4,1,0]heptane-2,3,4-triol. It is a unique pseudo-pyranose with a ß-epoxy moiety which corresponds to a carba analogue of ß-D-glucopyranose.[3]

Fig. II.4 Structure of cyclophellitol



The cyclophellitol was isolated from culture filtrate through charcoal separation, Dowex column chromatography and crystallisation.[3] After isolation and production of cyclophellitol from *Phellinus*, Astumi *et al* had determined the IC_{50} value, the concentration of the inhibitor exhibiting 50% inhibition on a specific enzyme. And the percentage inhibition was calculated by

$$\frac{\text{Abs (control)- Abs (test)}}{\text{Abs(control)}} \times 100\%$$

It inhibited almond-derived ß-D-glucosidase with an IC_{50} of 0.8 μg/ml.[2-3,43,50,52-54,59] This value was lower than the IC_{50}s of 1-deoxynojirimycin(30 μg/ml) and that of castanospermine (12 μg/ml).[3] The inhibition mechanism of cyclophellitol was still unknown. As expected, it should be a competitive inhibitor due to the structure similarity.

In 1990, Atsumi *et al* tested the cyclophellitol against nine glycosidases. It was inactive against yeast α-glucosidase, ß-galactosidase, ß-glucuronidase, α-L-fucosidase, end-ß-N-acetyl glucosaminidase, α-mannosidase, and cellulase but weakly active against fungal ß-xylosidase. However, it showed marked inhibition on almond ß-glucosidase. Inhibition was shown to be competitive type which was evidenced by Lineweaver-Burk plot. (Fig. II.5) The y-intercepts, $1/V_{max}$, of different concentrations of cyclophellitol were the same but the x-intercepts were different (1/Km). Only pure competitive inhibition showed this pattern. At high substrate concentration, the substrate wins the competition for the active site and the maximum velocity of the enzymatic

activity would not change. On the other hand, the substrate specificity (shown by Km) would decrease due to the presence of inhibitor. They also found out that long preincubation could enhance the inhibition on the enzyme. Irreversible binding studies were then carried out. Cyclophellitol-treated almond ß-glucosidase was equally suppressed after dialysis; thus cyclophellitol was likely to bind the enzyme irreversibly.[2]

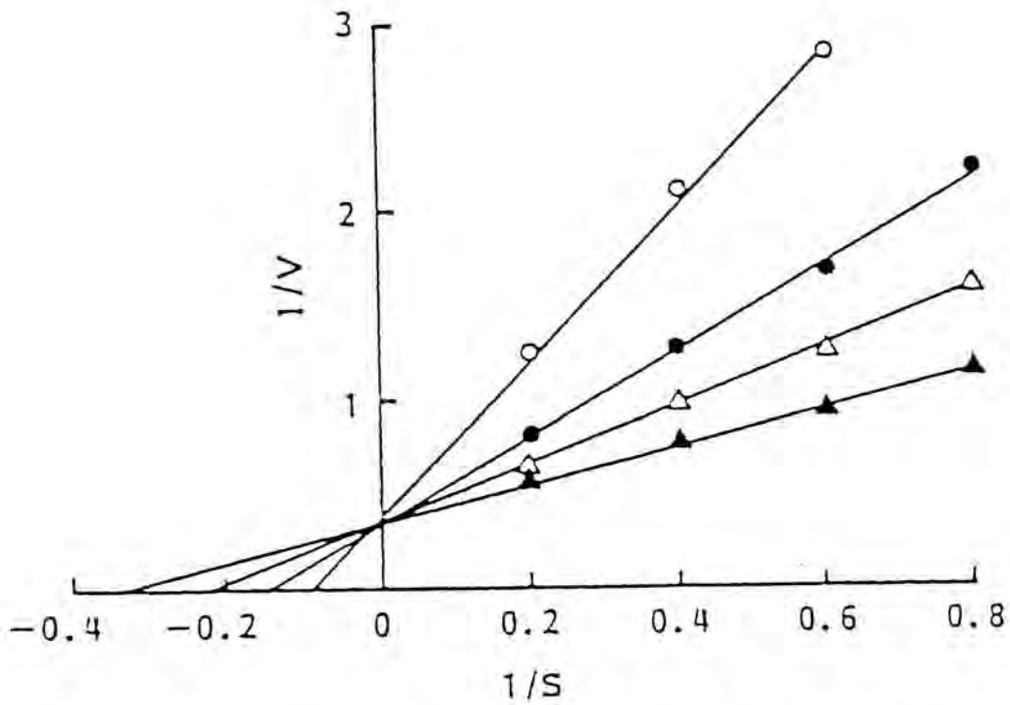


Fig.II.5 Lineweaver-Burk plot of almond β-D-glucosidase reaction with cyclophellitol, ○ 10 μM inhibitor, ● 5 μM, △ 2.5μM, ▲ control.

In addition, the inhibitor was found by fluorometric assay to be active against human ß-glucosidase but inactive toward α-glucosidase in Molt-4 microsomal fraction. It also inhibited Molt-4 ß-glucocerebrosidase completely at 2 μg/ml when the enzyme was assayed with a synthetic labelled substrate, and the inhibitory activity was more than one hundred times higher than that of nojirimycin, castanospermine, or of deoxynojirimycin. Although, cyclophellitol did not show cytotoxicity in cultured NIH3T3, P388 and Molt-4 cells even at 100 μg/ml, it showed severe toxicity in mice. Mice administered 1 mg of cyclophellitol daily for 5 days began to exhibit severe abnormalities of nervous system similar to those found in GAUCHER's mouse through inhibition of glucocerebrosidase activity.[2]

II.5.2 Mode of inhibition of cyclophellitol[59]

In 1991, the inhibitory action of cyclophellitol was characterized. It was indeed a specific covalent mechanism based inactivator which operated via the kinetic mechanism like conduritol epoxide. The compound bound at the active site in similar mode to the glucoside substrate, the epoxide oxygen was protonated by the acid catalytic group, then the nucleophilic carboxylate attacked this activated species forming a covalent derivative and inactivating the enzyme (Fig. II.6). Cyclophellitol was shown to be an excellent time-dependent inactivator of both the almond ß-D-glucosidases and *Agrobacter sp.*

ß-D-glucosidase with inactivation constants of Ki=0.34 mM, ki=2.38 min^{-1} (Fig. II.7), and Ki=0.055 mM , ki=1.26 min^{-1} respectively (Fig.II.8)

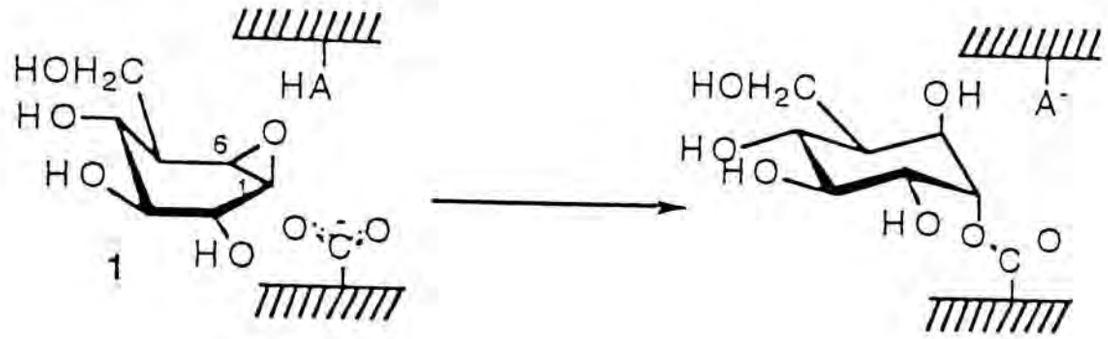


Fig. II.6 Proposed reaction of cyclophellitol with ß-glucosidase

Recently, cyclophellitol was being synthesised in laboratories for detailed biological evaluation and studies. Recent studies have revealed several synthetic pathway of cyclophellitol starting from L-glucose, from L-quebrachitol and from Diels-Alder adduct. In the Chemistry Department of CUHK, (-)-quinic acid was used as homochiral precursor in organic synthesis of cyclophellitol and its diastereoisomers.[43] The structure-function relationship of these compounds will be discussed in detail based on the biological assay in this study (Chapter III.1).

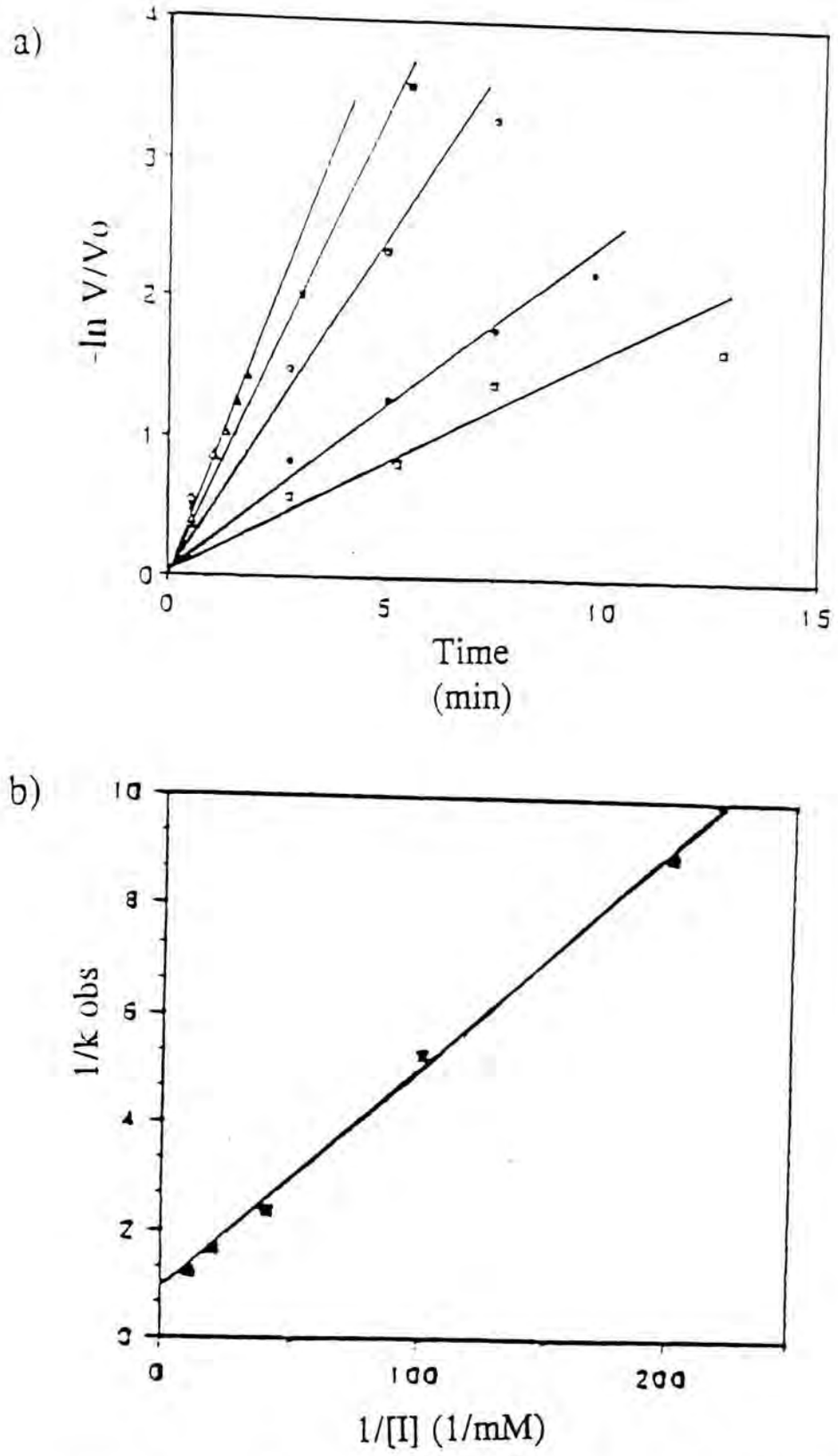


Fig. II.7 Inactivation of *Agrobacter* β-D-glucosidase by cyclophellitol.
a) Plot of ln residual activity ratio *versus* time; Concentrations of cyclophellitol employed were; (Δ) 0.1 mM, (■) 0.05 mM, (○) 0.025 mM, (●) 0.01mM, (□) 0.005 mM.
b) Replot of first order rate constants from a).[59]

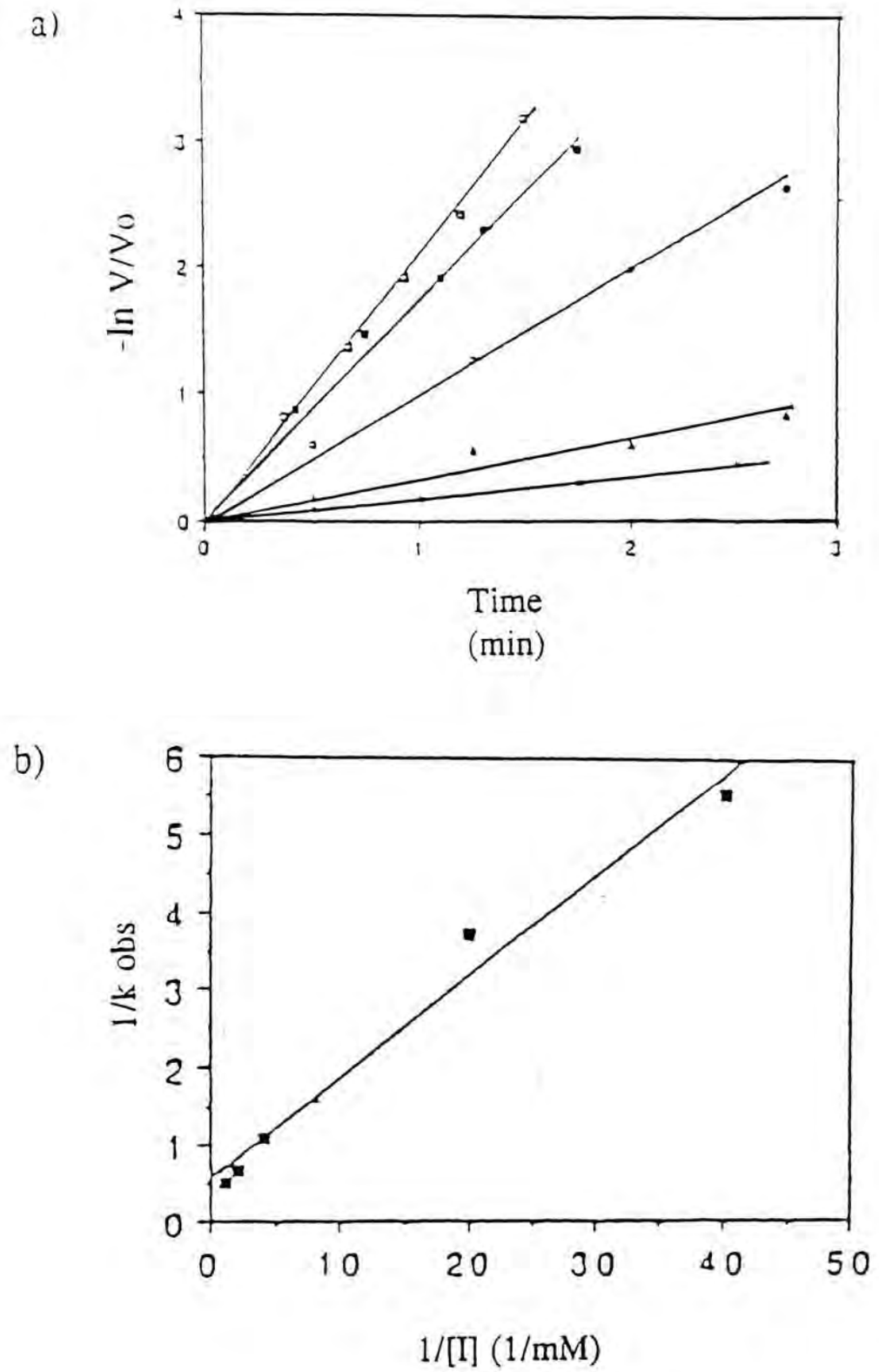
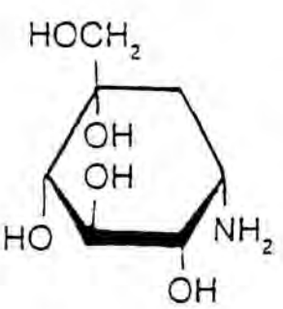


Fig. II.8 Inactivation of almond β-D-glucosidase by cyclophellitol.
a) Plot of ln residual activity ratio *versus* time; Concentrations of cyclophelltiol employed were : (□) 0.9 mM, (■) 0.5 mM, (○) 0.25 mM , (▲) 0.05 mM, (△) 0.025 mM.
b) Replot of first order rate constants from a).[59]

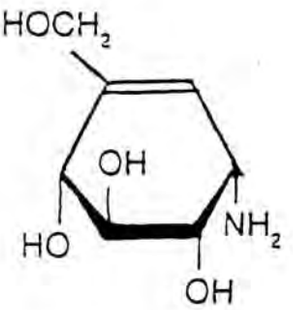
II.5.3 General background on aminocyclitols

Investigation on the aminocarbasugars was stimulated by the unusual properties of glycosidase inhibition found in the antibiotic validamycins in the fermentation broth of *Streptomyces hygroscopicus*. Some naturally occurring aminocarbasugars are readily obtained by degradation of this antibiotic or directly isolated from fermentation broth. They are known as validamine, valiolamine, hydroxyvalidamine and valienamine. They both have an amino-group at C-1 position and have similar hydroxylation pattern corresponding to that of D-glucose.

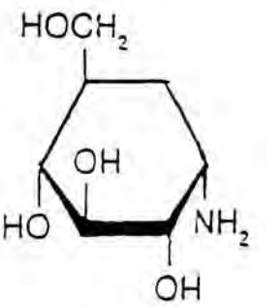
Fig.II.9 Structure of valiolamine, valienamine, validamine and hydroxyvalidamine



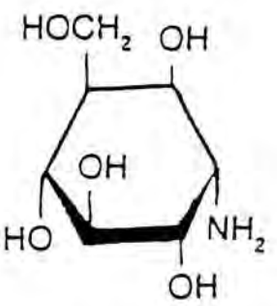
Valiolamine



Valienamine



Validamine



Hydroxyvalidamine

All of the aminocarbasugars were found to possess inhibitory activity against one or more glycosidases. Valiolamine was found to be considerably more active than the others. It had a potent α-glucosidase inhibitory activity against porcine intestinal sucrase, maltase and isomaltase.[24,49] Except for strong inhibition against the above enzymes, all of these compounds were much less potent inhibitors towards α- and ß-D-glucosidases than the inhibitors D-glucosylamine and 1-deoxynojirimycin. Replacement of the ring-oxygen or nitrogen atom by a $-CH_2-$ or $=CH-$ group thus appears to be detrimental to an efficient interaction with α- and ß-D-glucosidases. This could be explained by an adaptation of the active site favouring a half-chair conformation of the D-glucosyl residue. The inhibitors having an oxygen or nitrogen atom in the ring might better adapt to this geometry than the more rigid cyclohexane or cyclohexene.[32] The aminocarbasugars are actually reversible competitive in character. The competitive mode of inhibition was characterized by L-B plot [valienamine on yeast α-glucosidase (Fig. II.10) and valiolamine on porcine intestinal α-glucosidase (Fig. II.11)]. Unlike cyclophellitol, cyclohexane or cyclohexene with primary amine structure of these compound have no covalently interaction with the enzyme during inhibition. They are only transient associated with the enzyme.

The aminocyclitols, validamine, valiolamine and valienamine were also found to have inhibitory effects on oligosaccharide glucosidases I and II and on lysosomal α-glucosidase from rat liver.[51] These enzymes are involved in glycoprotein processing and carbohydrate metabolism in liver. Valiolamine again proved more potent an inhibitor than the others. Thus, these compounds have served as useful tools in investigations of carbohydrate metabolism.

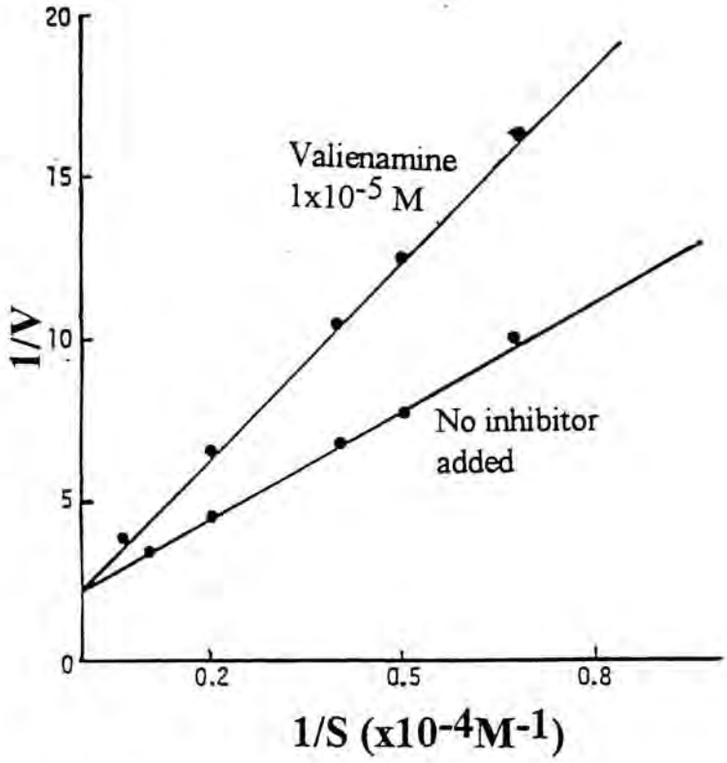


Fig. II.10 Effect of valienamine on hydrolysis of *p*-nitrophenyl-α-D-glucosidase by yeast-α-D-glucosidase.[25]

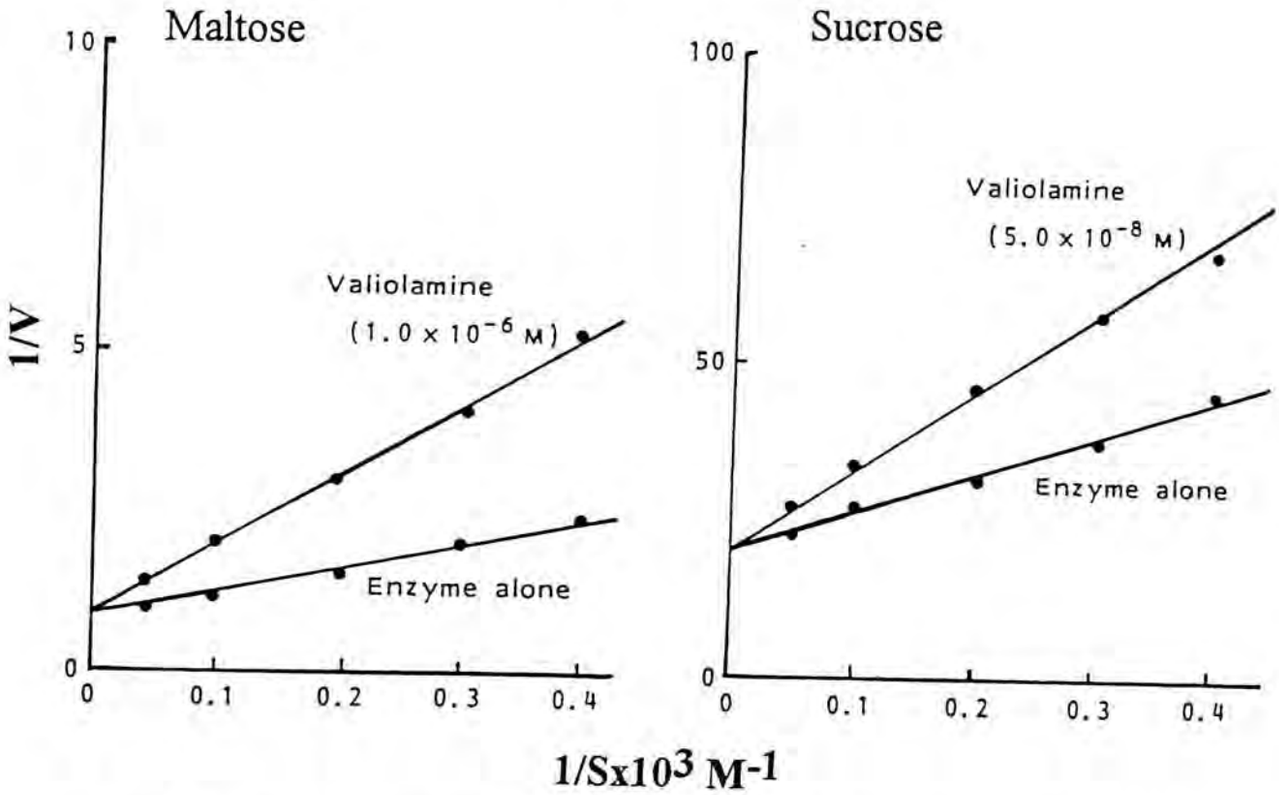


Fig. II.11 Effects of valiolamine on hydrolysis of maltose and sucrose by porcine intestinal α-D-glucosidases.[24]

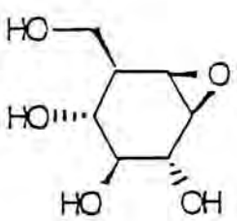
CHAPTER III: Characterization of Synthetic Glycosidase Inhibitors

III.1 Covalent-based Inactivator (Cyclophellitol and its Analogues)

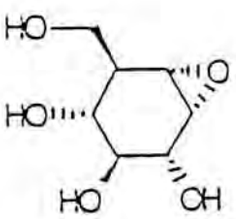
III.1.1 Introduction

In order to provide additional insight into the mode of action of glycosidase inhibition by cyclophellitol-like compounds, the glycosidase inhibiting activities of cyclophellitol and its unnatural (*1R,6S*)-, (*2S*)- and (*1R,2S,6S*)-diastereoisomers, as well as two 6-deoxy-1,2-epoxy analogues were described.

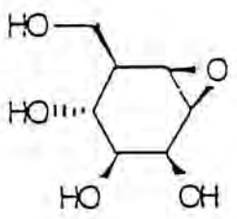
Fig.III.1 Structure of cyclophellitol and its analogues



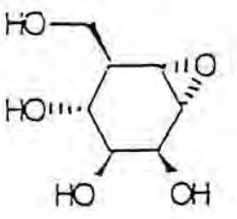
Cyclophellitol



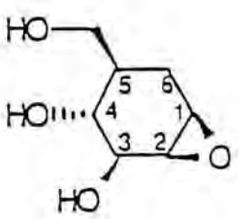
(1R,6S)-diastereoisomer



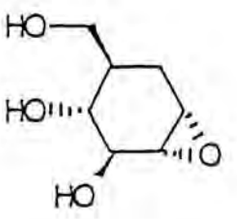
(2S)-diastereoisomer



(1R,2S,6S)-diastereoisomer



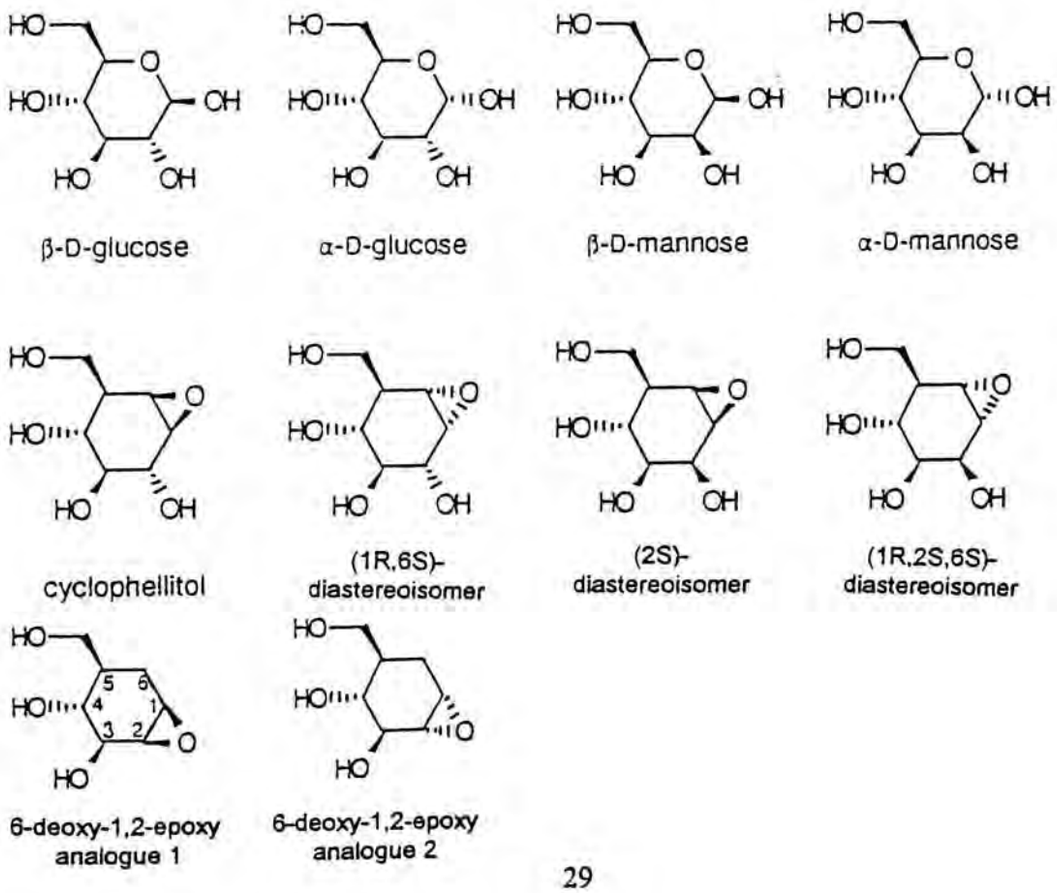
6-deoxy-1,2-epoxy analogue 1



6-deoxy-1,2-epoxy analogue 2

The diastereoisomers tested have different configurations of the epoxide ring at C-1,6 and of the hydroxy group at C-2. The two 6-deoxy-1,2-epoxy analogues have the oxirane shifted to another position, C-1,2. Each of them resembles a glycoside and is expected to inhibit the corresponding glycosidase. The epoxide, the three hydroxy groups, and the hydroxymethyl group in cyclophellitol constitute the ß-D-*gluco*-configuration. Cyclophellitol had been reported to be a mechanism-based active site directed inhibitor of almond ß-D-glucosidase (section II.5.2). The unnatural (*1R,6S*)-, (*2S*)- and (*1R,2S,6S*)-diastereoisomers having the α-D-*gluco*-, ß-D-*manno*- and α-D-*manno*-configurations should be expected to inhibit the configurationally related glycosidases (Fig. III.2).

Fig. III.2 Structure comparison between sugars and cyclophellitol & its analogues



The glycosidase inhibiting activities are assayed first to find out which enzyme is inhibited by each compound. The IC_{50} value is obtained from the inhibition-concentration curve to reflect the effectiveness or potency of each compound as an inhibitor.

Cyclophellitol had already been characterized as an irreversible mechanism-based inactivator (II.5.2). The parameters, inactivation constant Ki and rate constant ki were reported. However, the inhibition mechanism of the other synthesized analogues have not been determined yet. It is expected that they inhibit the enzyme in a similar manner like the parent compound, cyclophellitol. Thus, studies on irreversible binding and kinetic analysis were conducted on its analogues.

For kinetic studies, the method for analysing results was devised by Kitz and Wilson.[28,33] The enzyme inactivation was believed to proceed by a two-step mechanism. In the first step, the epoxide is protonated by a general acid catalytic group to form a reversible, non-covalent E-I complex. This step is characterized by the dissociation constant Ki, representing the affinity between the E-I complex. In the second step, this non-covalent complex is being activated and attacked by a carboxylate group of an amino acid (a.a.) at the active site. Covalent bond is subsequently formed between the inhibitor and a.a. residue at the active site. This second, rate limiting step is

characterized by the rate constant ki, relating to the half life of the E-I complex.

The whole reaction scheme is illustrated as below.

$$E + I \underset{}{\overset{Ki}{\rightleftharpoons}} EI \xrightarrow{ki} EI^*$$

When the logarithm of the residual activity is plotted versus time, a linear plot is obtained. The slope of this line provides the pseudo-first order rate constant for inactivation at each inhibitor concentration.

$$\ln\frac{E}{E_o} = -k_{app}.t$$

E = residual enzyme activity at time t
E_o = initial enzyme activity
k_{app} = experimental rate constant
t = time

$$1/k_{app} = Ki/ki \cdot 1/I + 1/ki$$

A replot of the reciprocal of these rate constant (k_{app}) versus reciprocal inactivator concentration (I) yields a straight line giving a value of y-intercept $=1/ki$ and slope, $m = Ki/ki$. The two inactivation parameters, Ki and ki can then be determined from the graph.[22,28,30,33,55,60]

Actually, these two values are more better indicators to reflect the potency of the inhibitor compared with IC_{50}.

III.1.2 Materials

Instrumentation: Only continuous assay of enzymatic activity in kinetic studies was measured on the Spectronic 3000 array spectrophotometer. Other measurements were recorded on the 601 spectrophotometer.

Assay of glycosidase activity: The substrates, *p*-nitrophenyl glycosides, were purchased from Sigma Chemical Company, as were the α-D-glucosidase (Type VI: from Brewers yeast), ß-D-glucosidase (from almonds), α-D-mannosidase (from jack beans), ß-D-mannosidase (from snail acetone powder), α-D-galactosidase (from *Escherichia coli*) and ß-D-galactosidase (from *Aspergillus oryzae*). Another source of ß-D-mannosidase was partially purified from the mycelia of *Aspergillus oryzae,* ATCC 14895 as described in the text.

Dialysis: Molecular porous dialysis membrane MWCO: 2,000 (wet in 0.05 % sodium azide) was purchased from Spectrum Company.

Protein assay in purification of ß-D-mannosidase: Bicinchoninic acid protein assay kit with Sigma procedure no. TPRO-562 (For kit no. BCA-1 and Product no. B-9643) and albumin stock solution (905-10) were purchased from Sigma Chemical Company.

III.1.3 Methods

The inhibition assays on six commercially available glycosidases are described in the following sections:

III.1.3.1 Inhibitory assay of commercially available glycosidases

The enzyme activities were determined according to the method described by *Saul* with slight modification.[41] The reaction mixture contained 20 mM of the appropriate buffer, 5 mM *p*-nitrophenyl glycoside ,the inhibitor and an enzyme in a final volume of 0.5 ml. Enough enzyme was used to give an absorbance value about 1.0 within 10 min. Control was included by replacing the inhibitor in the reaction mixture with water. After incubation at 30 °C for a definite period of time, 2.5 ml of 0.4 M glycine-NaOH buffer (pH 10.0) was added to quench the reaction and liberated *p*-nitrophenol was measured at 410 nm (ϵ_{410} 17,000 L mol^{-1} cm^{-1}). No preincubation of the inhibitor-enzyme mixture was conducted unless stated otherwise in the text. The following table shows the buffer, pH values and substrates for six commercially available enzymes. In addition, the enzyme units in final concentration of reaction mixture are also listed.

Table III.1 The assay conditions (enzyme unit, substrate, pH and the buffer composition used) for inhibition of six different glycosidases by cyclophellitol and its analogues

Enzyme	Enzyme unit (mU/ml)	Substrate	pH value	Buffer
α-D-glucosidase (Brewers yeast)	250	*p*-nitrophenyl α-D-glucopyranoside	6.0	Phosphate
ß-D-glucosidase (almonds)	50	*p*-nitrophenyl ß-D-glucopyranoside	5.0	Sodium acetate
α-D-mannosidase (Jack beans)	62.5	*p*-nitrophenyl α-D-mannopyranoside	4.5	Sodium acetate
ß-D-mannosidase (Snail acetone powder)	25	*p*-nitrophenyl ß-D-mannopyranoside	4.0	Sodium acetate
α-D-galactosidase (*E. coli*)	50	*p*-nitrophenyl α-D-galactopyranoside	6.5	Phosphate
ß-D-galactosidase (*A. oryzae*)	50	*p*-nitrophenyl ß-D-galactopyranoside	4.5	Sodium acetate

One unit of enzyme activity is defined as that catalysing the conversion of 1 μmole substrate (or the formation of 1 μmole product) in 1 minute.[12]

III.1.3.2 Partial purification of ß-D-mannosidase from *A. oryzae*[31,36]

The spore suspensions of *A. oryzae* (0.5 ml) in each culture flask were inoculated with 125 ml potato dextrose broth (24 g/L) supplemented with glucose (5 g/L). Cultivation was carried out for 3.5 days at 28 °C under shaking. 49.21 g of mycelia (wet weight) were obtained from 2 litres of medium. The mycelia were homogenized with sand in 37 ml cold sodium acetate buffer at pH 4.0. The mixture was centrifuged at 27500 xg for 20 min at 4 °C to obtain the supernatant as the crude preparation. 49 ml supernatant was fractionated at 70-95% ammonium sulphate precipitation. The protein

pellet obtained after centrifugation at 20200 xg was redissolved in 2.0 ml 100 mM sodium acetate buffer (pH 4.0). After dialysis against 10 mM of the same buffer, the dialysed extract were chromatographed on 43.4 ml cation exchanger, CM-Sepharose CL-6B (Pharmacia) with UV/VIS detector (280 nm, ISCO UA-6).[26,40] It was equilibrated first with the 10 mM sodium acetate buffer (pH 4.0). The flow rate was 2.0 ml per min. After loading the sample, 80 ml of 10 mM sodium acetate buffer was used to elute the unbound proteins. The bound proteins were eluted with stepwise gradient of 0.1 to 0.5 M sodium chloride in the same buffer. The fractions having ß-D-mannosidase activity were mainly collected at 0.2 M sodium chloride solution (see the elution profile shown in Fig. III.3). All the active fractions were pooled and dialysed. Finally, the sample after lyophilization, 10.8 mg was used as stock enzyme source for inhibition assay. The fold of purification in each purification step was summarised in Table III.2. The enzyme was purified approximately to 55-fold compared to the crude preparation.

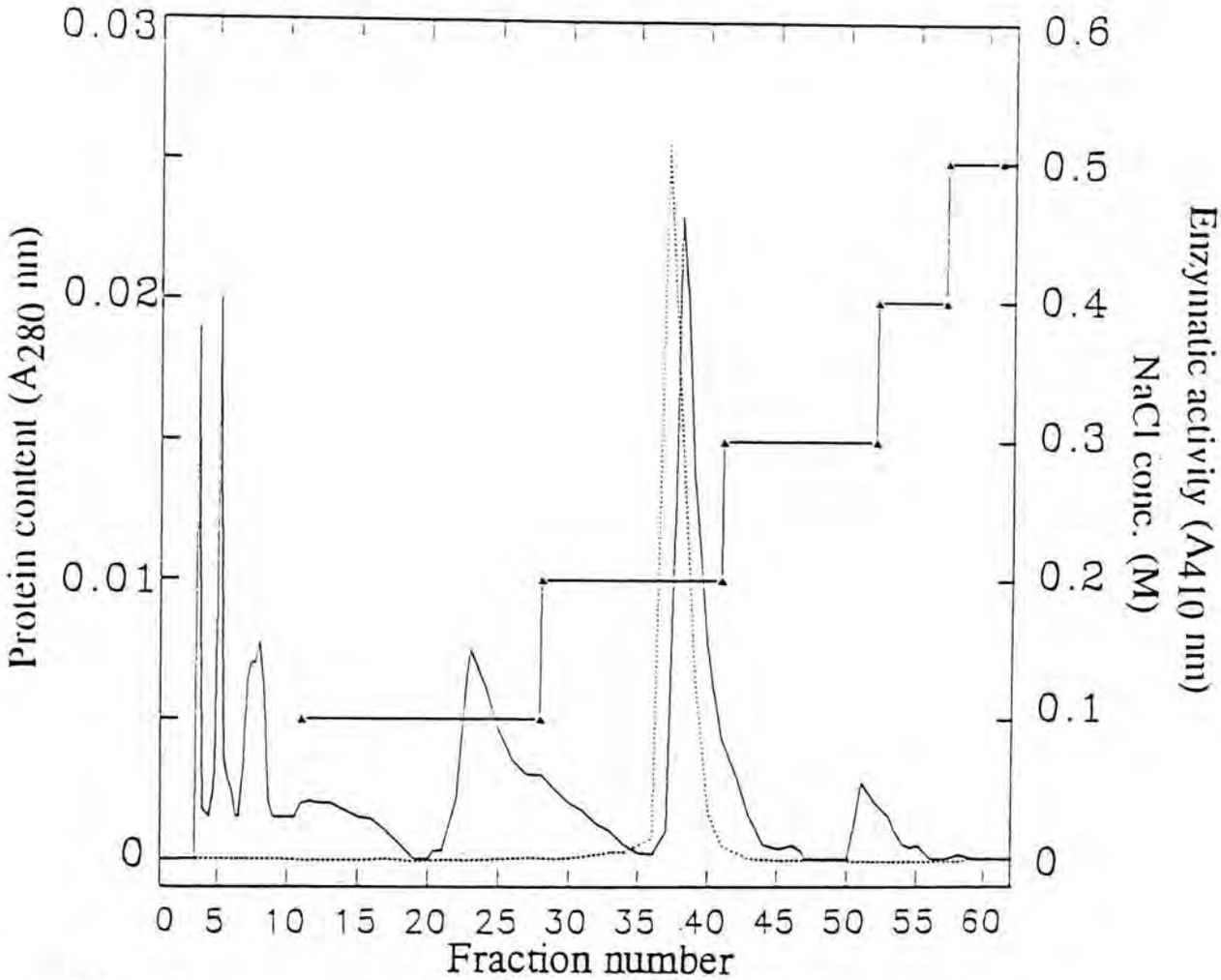


Fig. III.3 Chromatography of the partially purified β-D-mannosidase isolated from *A. oryzae* on CM-Sepharose CL-6B after ammonium sulphate precipitation (70-95 satn.) Fraction 11-52 were 4 ml each and the others were 8 ml. Protein content A_{280} nm (——); enzymatic activity, A_{410} nm (.....); NaCl (▲—▲)

Table III.2 A summary of data on purification of β-D-mannosidase from *A. oryzae*.

	Protein content (mg)	Total activity (μmoles/min)	Specific activity (μmoles/mg /min)	Purification (fold)
Crude enzyme	120.6	0.7987	6.62×10^{-3}	1
$(NH_4)_2SO_4$, 0.7-0.95 satn.	2.456	0.3012	1.23×10^{-1}	18.6
CM-Sepharose chromatography	0.4896	0.1786	3.65×10^{-1}	55.0

III.1.3.3 Protein assay in purification of ß-D-mannosidase

Protein concentrations of the samples were determined by the method of Bicinchoninic acid protein assay (BCA)[46].

Bicinchoninic acid solution, reagent A and Copper(II) sulfate pentahydrate 4 % solution, reagent B were obtained from BCA assay kit. The protein determination reagent was freshly prepared by adding 1 part of reagent A to 50 parts of reagent B. Protein standard solution (1 mg/ml) was diluted from albumin stock solution (10 mg/ml).

A series of standard solutions of 0, 0.02, 0.04, 0.06, 0.08, 0.10 ml protein standard solution were prepared. Each tube was made to 0.10 ml by adding water. The test sample added was also 0.1 ml. If the absorbance of the sample was out of the range of standard curve prepared, dilution was needed and counted. 2.0 ml of the protein determination reagent was added to each tube and vortex. After incubation for 30 min at 37 °C, the absorbance at 562 nm was determined after cooling the tube to room temperature.

III.1.3.4 Inhibitory assay for partially purified ß-D-mannosidase(*A. oryzae*)

The enzyme was prepared according to the above purification step. The (2*S*)-diastereoisomer was pre-incubated with the enzyme in sodium acetate buffer (pH=4.0) for 15 min at 30 °C. The reaction was started by the addition of 0.2 ml *p*-nitrophenyl-ß-D-mannopyranoside. The reaction mixture contained a final concentration of 20 mM sodium acetate buffer, 5 mM substrate and ß-

D-mannosidase (15.28 mU/ml, specific activity 0.172 U/mg) and the inactivator in 0.5 ml final volume. After incubation for 20 min at 30°C, 2.5 ml of 0.4 M glycine-NaOH buffer (pH=10.0) was added to stop the reaction and the liberated *p*-nitrophenol was measured by spectrophotometer at 410 nm (17,700 L $mol^{-1}cm^{-1}$).

Enzyme assay for 6-deoxy-1,2 epoxide analogue 1 against partially purified ß-D-mannosidase was the same as for (*2S*)-diastereoisomer. The preincubation time was 15 min and the reaction time was 30 min.

III.1.3.5 Influence of dialysis on glycosidase inhibition[2]

Various concentrations of inhibitor or product (glucose and mannose) (0.5 ml) was mixed with 0.5 ml of 100 mM corresponding buffer (listed in Table III.1) and 1 ml of enzyme solution (2.5 U/ml for both α-glucosidase and α-mannosidase) and incubated for 11 hours at 4 °C. One half volume was dialysed against 10 mM of same buffer, dialysed sample and the rest was stood at 4 °C for 11 hours, control sample. For α-mannosidase, the control and the dialysed sample had to be diluted ten fold prior to performing the assay. The assay reaction was then carried out similar to those mentioned before. (III.1.3.1)

III.1.3.6 Inactivation experiment on glycosidases[6,59]

The inactivation was performed by incubating the enzyme in the buffer with the presence of inactivator. Aliquot (10 μl) was removed at appropriate time intervals and assayed for residual enzyme activity by dilution into a large

volume (1.49 ml) of saturating concentrations of the corresponding *p*-nitrophenyl glycoside substrate in the same buffer system. This effectively halts the inactivation both by diluting the inactivator enormously, and by providing high concentrations of a competitive ligand, the substrate. Activity was determined by continuous monitoring of nitrophenolate released through increase in absorbance at 400 nm. Buffer systems, temperatures and substrates employed for the inactivation experiments for each enzyme were as follows: Brewers yeast α-D-glucosidase, 50 mM sodium phosphate buffer (pH=6.8), 25 °C, *p*-nitrophenyl α-D-glucopyranoside; Jack beans α-D-mannosidase, 50 mM sodium phosphate buffer (pH=6.0), 25°C, *p*-nitrophenyl α-D-mannopyranoside.

III.1.4 Results

III.1.4.1 Inhibitory activities of cyclophellitol and its analogues against glycosidases

Each compound was preliminary screened for six glycosidases. The method was described in II.1.3.1. All the data are shown in Table III.3.[50]

Table III.3 Inhibitory activities of cyclophellitol and its analogues against six glycosidases in the preliminary screenings.

Enzyme	% Inhibition					
	Cyclophellitol	(1R,6S)-	(2S)-	(1R,2S,6S)-	6-deoxy-1,2-epoxy analogue 1	6-deoxy-1,2-epoxy analogue 2
α-D-galactosidase (*E. coli*)	2[b]	18[c]	6[d]	17[e]	12[f]	2[f]
β-D-galactosidase (*A. oryzae*)	3[b]	19[c]	15[d]	11[e]	7[f]	0[f]
α-D-glucosidase (brewers yeast)	0[b]	47[c]	0[d]	13[e]	0[e]	0[e]
β-D-glucosidase (almonds)	99[b]	15[c]	3[d]	7[e]	83[e]	3[e]
α-D-mannosidase (jack beans)	0[b]	2[c]	12[d]	100[e]	0[e]	0[e]
β-D-mannosidase[a] (*A. oryzae*)	3[e]	2[e]	30[e]	3[e]	12[e]	2[e]

[a]Purification up to ammonium sulphate precipitation stage for preliminary screening. [b]Inhibition at the final concentration of 40 μg/ml. [c]Inhibition at the final concentration of 80 μg/ml. [d]Inhibition at the final concentration of 200 μg/ml. [e]Inhibition at the final concentration of 100 μg/ml. [f]Inhibition at the final concentration of 160 μg/ml.

Table III.4 indicates the enzyme specificity of each compound and the IC_{50}s obtained by us and by Umezawa group.[2,3,52-54]

Table III.4. The enzyme specificity and IC_{50} of cyclophellitol and its analogues on glycosidases

Inhibitors	Enzyme Inhibited	IC_{50} (μg/ml)
Cyclophellitol	ß-D-glucosidase (50 mU/ml)	0.35 *0.80
(*1R,6S*)-diastereoisomer	α-D-glucosidase (250 mU/ml)	24 *10
(*2S*)-diastereoisomer	partially purified ß-D-mannosidase from *A. oryzae* (15.3 mU/ml)	28
(*1R,2S,6S*)-diastereoisomer	α-D-mannosidase (25 mU/ml)	9.0 *19
6-deoxy-1,2-epoxy analogue 1	ß-D-glucosidase (50 mU/ml)	50
	partially purified ß-D-mannosidase (8.70 mU/ml)	53
6-deoxy-1,2-epoxy analogue 2	-	-

* by Umezawa group[2,3,52-54]

Both values fall into the same order of magnitude. The small difference in data may be attributed to the different concentrations of enzymes used in the biological assay.

(2S)-Diastereoisomer was only weakly active towards the snail ß-mannosidase but inactive against the others at 100 μg/ml. However, 5.7% inhibition on snail ß-D-mannosidase was too low to be considered to have inhibitory effect on that enzyme. Since it is structurally related to ß-D-

mannose, it might be a potential ß-D-mannosidase inhibitor. The poor inhibition of this enzyme might be due to the enzyme source used. Although, the gut solution of snail is useful source of ß-mannosidase and the highly purified preparation of this enzyme can be obtained by simple chromatographic steps. The instability and low specific activity limit its action. Reports on the substrate specificity and kinetics of this enzyme are only restricted mainly to the action on core glycopeptides and derived oligosaccharides.[36]

In previous studies of glycosidase inhibition, ß-D-mannosidase used was not purchased commercially. One source of ß-D-mannosidase was isolation from spray-dried culture filtrate of *Aspergillus wentii*.[31] In this work, two species of Aspergillus , *A. niger* and *A. oryzae*, were screened for the presence of ß-D-mannosidase. The results showed that the culture filtrate and mycelia of both species had ß-D-mannosidase activity in the crude preparation. After a preliminary testing with (*2S*)-diastereoisomer as the inhibitor, mycelia of *A. oryzae* was chosen for the preparation and production of ß-D-mannosidase for detailed screening of all the compounds. The IC_{50} value of (*2S*)-diastereoisomer with partially purified ß-D-mannosidase from *A. oryzae* with 15.3 mU/ml is 28 μg/ml. This value falls into the range of other diastereoisomers.

III.1.4.2 Effect of dialysis on glycosidase inhibition

Our data show that the inhibitory activity of cyclophellitol on ß-D-glucosidase is not lost after dialysis, thus verifying the irreversible binding of this compound. This biological property was also demonstrated for the two (*1R, 6S*)-and (*1R, 2S, 6S*)-diastereoisomers. Fig. III.4a shows that the percentage inhibition of dialysed and control sample up to 4.0 μg/ml were nearly the same. Thus, dialysis did not reverse the binding between the enzyme and the inhibitor. This a kind of irreversible binding clearly differs from the glucose inhibition which is reversed by dialysis.(Fig. III.4b)

The (*1R, 2S, 6S*)-diastereoisomer proved much more inhibitory towards α-D-mannosidase, since a very low concentration (0.025 μg/ml) of inhibitor had already exhibited over 70 % inhibition (see Fig. III.5a). Both dialysed and control samples gave overlapping curves from 0.25 to 1 μg/ml. Similar to the ß-D-glucosidase experiments described above, little recovery of α-D-mannosidase activity was observed after extensive dialysis and the inhibitory action of the enzyme product (mannose) was reversed. (Fig. III.5a&b).

With regard to the (*2S*)-diastereoisomer, no investigation of irreversible binding was attempted due to limitation in the amount of enzyme available.

In conclusion, the results of the two diastereoisomers revealed that the whole series of cyclophellitol analogues shown in Fig. III.1 bind to the glycosidase in a mode similar to cyclophellitol itself.

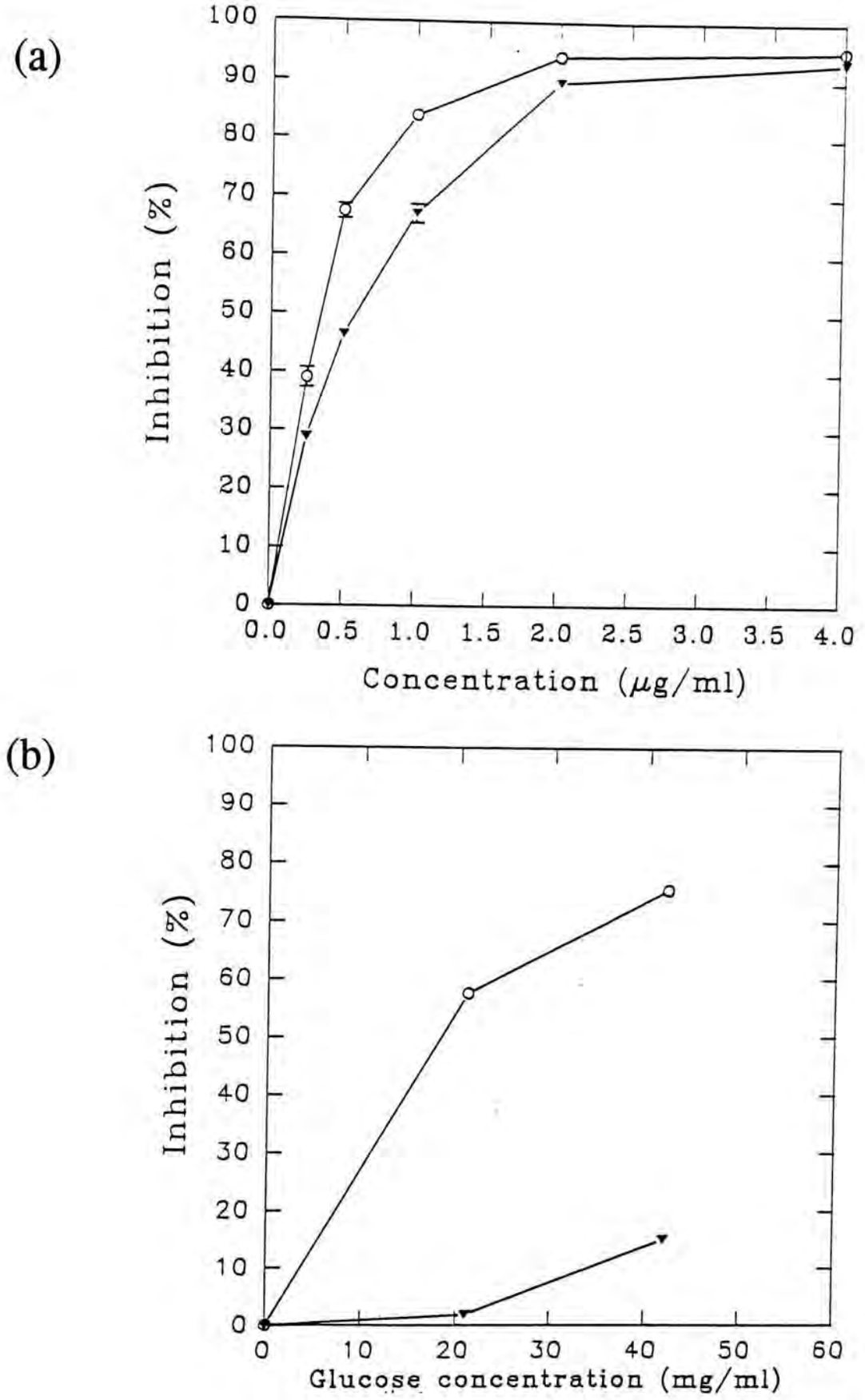


Fig. III.4 Effect of dialysis on the inhibition of brewers yeast α-D-glucosidase (250 mU/ml) by the (*1R,6S*)-diastereoisomer (graph a) or glucose (graph b). One half volume was dialysed for 11 hr. at 4 °C, dialysed sample (▼—▼). The rest was stood without dialysis, control sample (○—○).

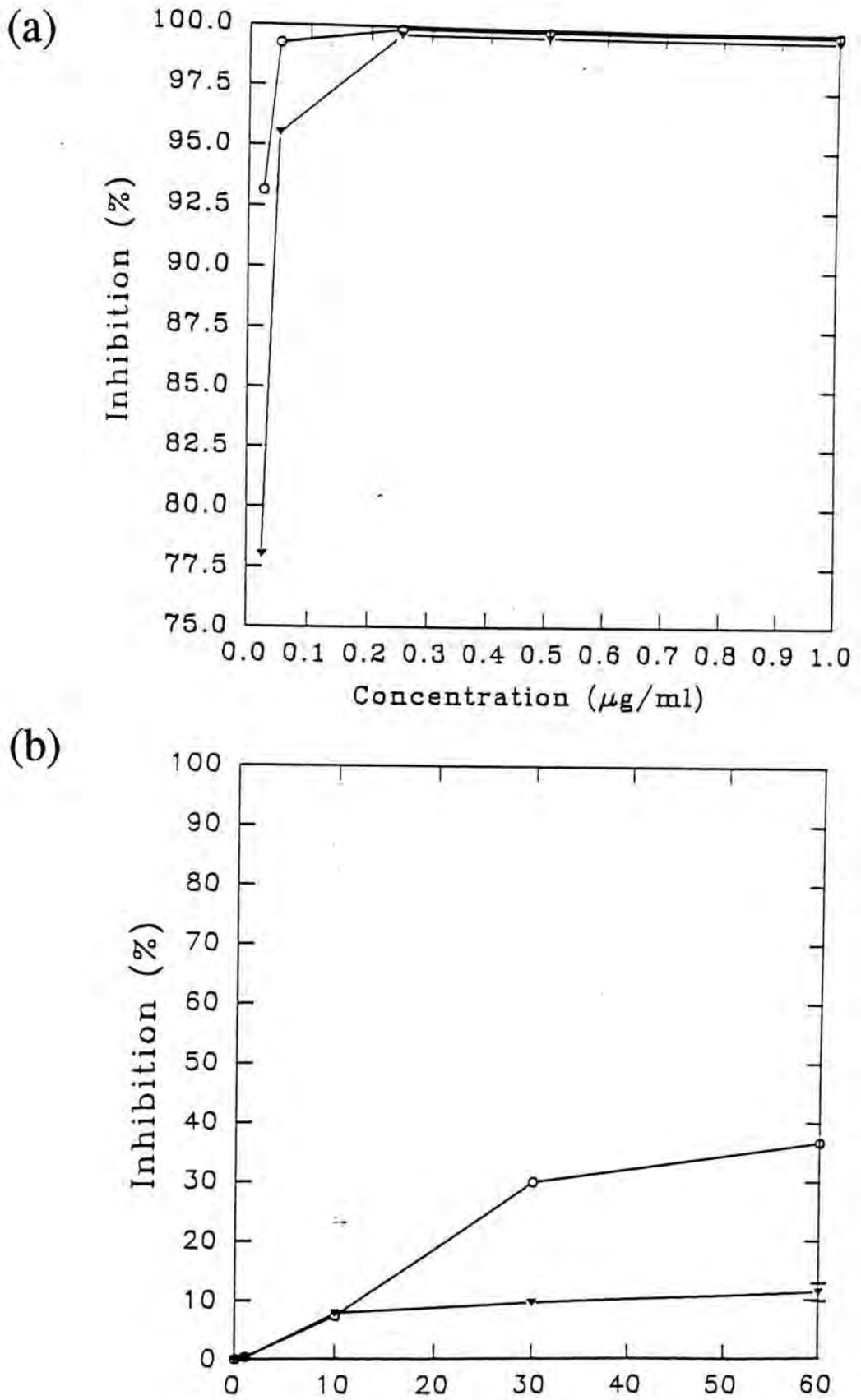


Fig. III.5 Effect of dialysis on the inhibition of jack beans α-D-mannosidase inhibition (50 mU/ml) by (*1R,2S,6S*)-diastereoisomer (graph a) or mannose (graph b). One half volume was dialysed for 11 hr. at 4 °C, dialysed sample (▼—▼). The rest was stood without dialysis, control sample (○—○).

III.1.4.3 The kinetic studies of glycosidase inactivation

We determined the kinetic parameters of inactivation based on the anticipated reaction scheme (said in III.1.1)

The inactivation of Brewers yeast α-D-glucosidase (266.7 mU/ml) by (*1R, 6S*)-diastereoisomer was presented in Fig. III.6a. All the plots of inhibitor concentrations with time were clearly linear as required for first order kinetic behaviour. The slopes (k_{app} values) obtained in this manner were then reploted according to the equation: $1/k_{app} = Ki/ki \cdot (1/I) + 1/ki$. A straight line was obtained with a rate constant, ki=0.401 min^{-1} and an equilibrium constant, Ki=25.6 μM. (Fig. III.6b)

(*1R, 2S, 6S*)-Diastereoisomer was also proved to be an inactivator of jack beans α-D-mannosidase. It showed the saturable first order kinetics of inactivation as expected for the inactivator which bind reversibly to the enzyme prior to covalent bond formation (Fig. III.7a). A replot of the reciprocal of rate constants versus reciprocal inactivator concentrations yielded the value of ki=2.85 min^{-1} and Ki=120 μM (Fig.III.7b). All the data in graph b were the means of duplicate trials.

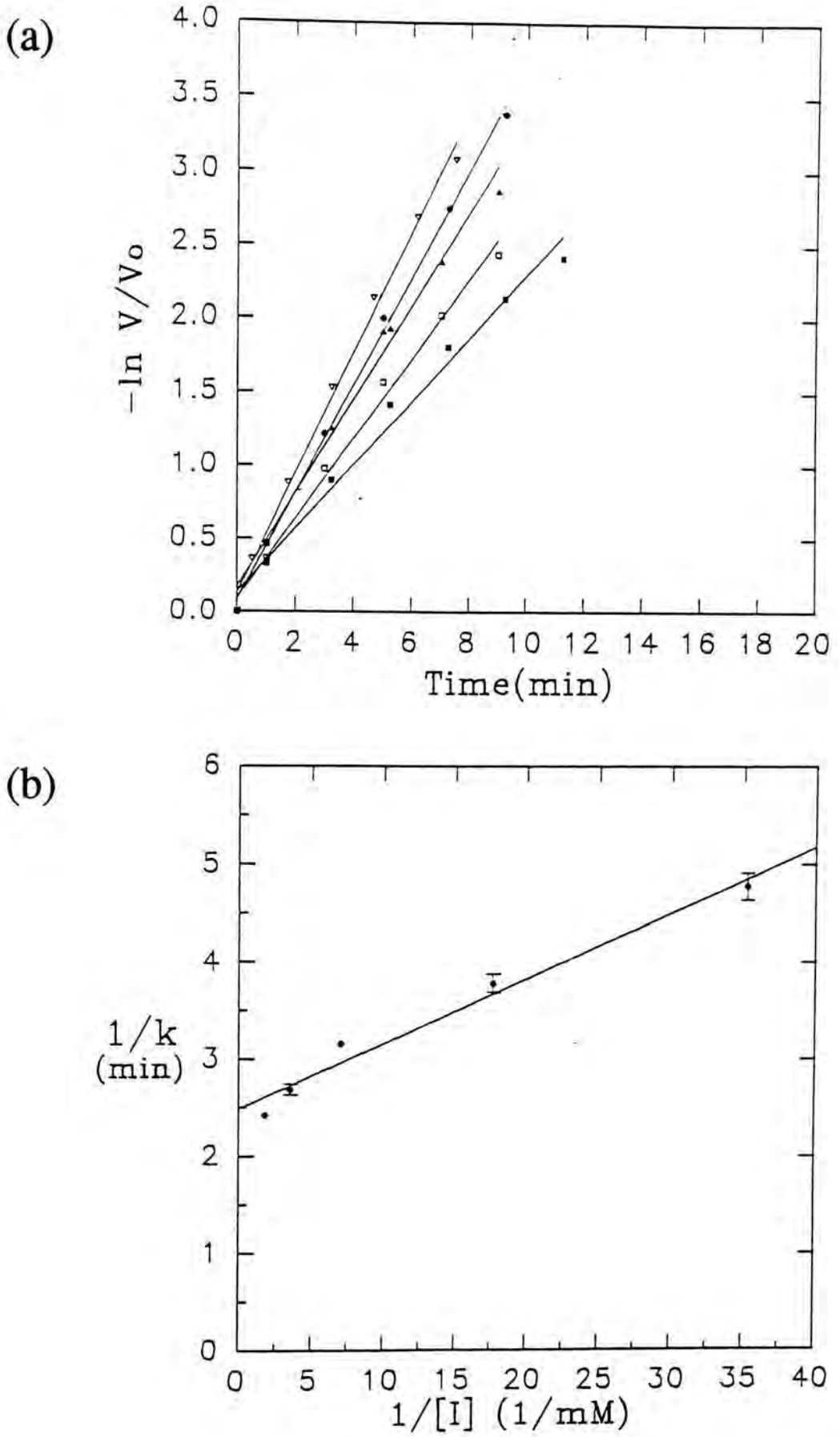


Fig. III.6 Inactivation of brewers yeast α-D-glucosidase, 266.7 mU/ml by (*1R,6S*)-diastereoisomer.
a) Plot of ln residual activity ratio versus time. Concentration of (*1R,6S*)-diastereoisomer employed were: (▽) 0.568 mM, (•) 0.284 mM, (▲) 0.142 mM, (□) 0.0568 mM, (■) 0.0284 mM.
b) Replot of first order rate constants from a).

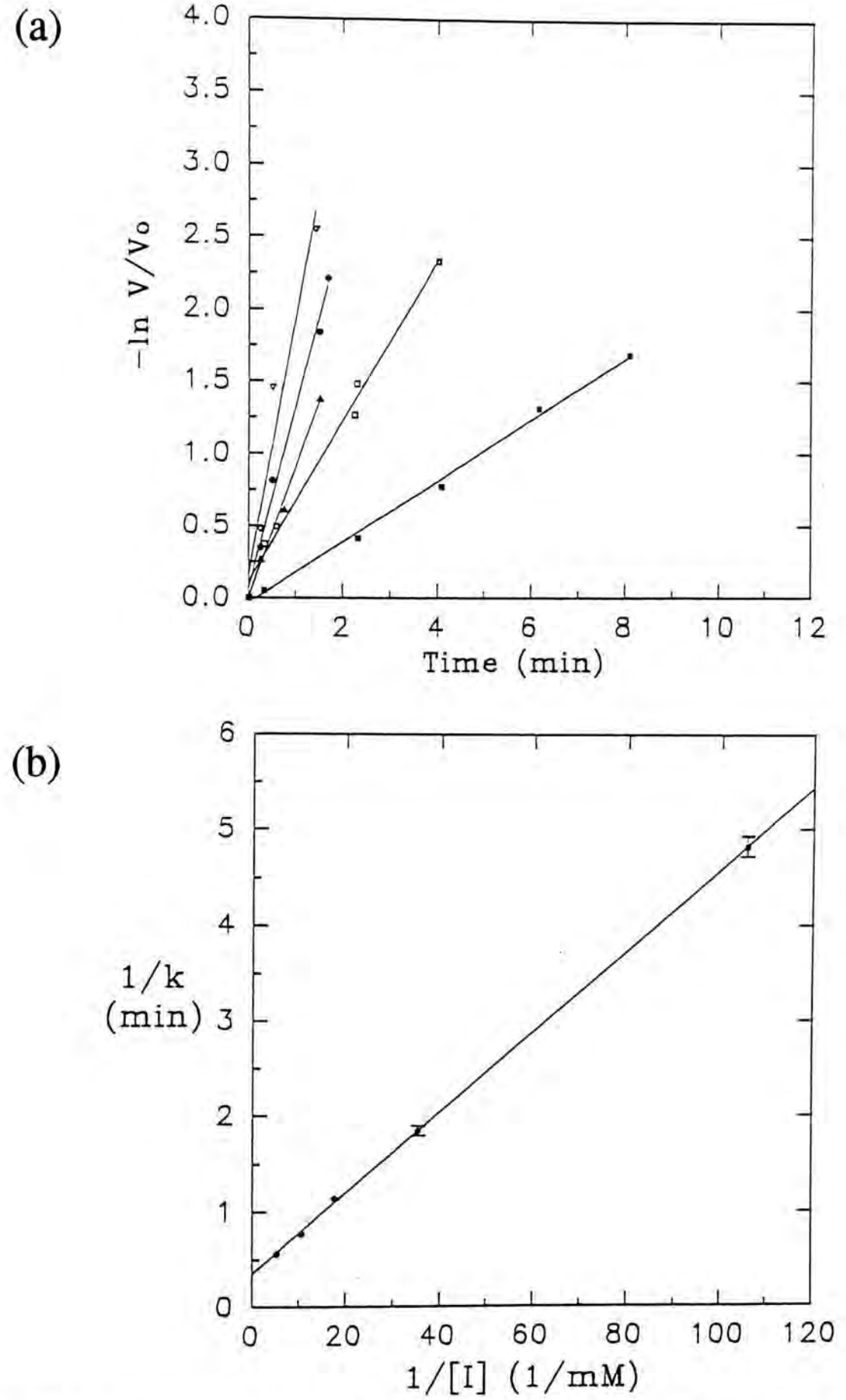


Fig. III.7 Inactivation of jack beans α-D-mannosidase, 50 mU/ml by (*1R,2S,6S*)-diastereoisomer.

a) Plot of ln residual activity ratio versus time. Concentration of (*1R,2S,6S*)-diastereoisomer employed were: (▽) 0.189 mM, (•) 0.0947 mM, (▲) 0.0568 mM, (□) 0.0284 mM, (■) 9.47 x 10^{-3} mM.

b) Replot of first order rate constants from a).

III.1.5 Discussion

Cyclophellitol and its analogues are expected to have a half-chair or sofa conformation since the X-ray crystallographic analysis of cyclophellitol has already indicated that it has a half-chair conformation[3]. Our assay results suggest that transition-state analogue inhibitors for glycosidases should possess a conformation close to that of a flattened chair of the glycopyranosyl cation together with the correct configuration at C-2, -3, -4, and -5.

Cyclophellitol is a potent inhibitor of almond ß-glucosidase. The unnatural (*1R,6S*)-, (*2S*)- and (*1R,2S,6S*)-diastereoisomers also showed strong and specific inhibition against α-D-glucosidase (brewers yeast), ß-D-mannosidase (*A. oryzae*) and α-D-mannosidase (jack beans) based on the results in Tables III.3 and III.4. The (*2S*)-diastereoisomer was determined to be a specific inhibitor of ß-D-mannosidase (*A. oryzae*) and 6-deoxy-1,2-epoxy analogue 1 was found to be an inhibitor for both ß-D-glucosidase (almonds) and ß-D-mannosidase (*A. oryzae*). The lack of a C-2 hydroxy group in 6-deoxy-1,2-epoxy analogue 1 results in non-specificity towards glucosidase and mannosidase. It is believed that the C-2 hydroxy group is important in recognition of the specific enzyme. The α-OH at C-2 defines the compound as a glucosidase inhibitor while ß-OH defines it as a mannosidase inhibitor. This was demonstrated by (*2S*)- and (*1R,2S,6S*)-diastereoisomers which have ß-OH at C-2 and inhibited ß-D- and α-D-mannosidases, respectively.

Concerning the stereochemistry of the oxirane moiety, cyclophellitol,

(*2S*)-diastereoisomer and 6-deoxy-1,2-epoxy analogue 1 which possess ß-epoxides, are ß-D-glycosidase inhibitors, whereas (*1R,6S*)- and (*1R,2S,6S*)-diastereoisomers which possess α-epoxides, are α-D-glycosidase inhibitors. Thus, the synthetic inhibitors are glycosidase-specific with respect to the epoxide stereochemistry. Since, it was found that the oxirane ring of conduritol epoxides were opened regiospecifically at C-1[32], we envisaged that, by analogy, the epoxide in cyclophellitol would also be opened by ß-D-glucosidase regiospecifically at C-1. As illustrated in Fig. II.7, the oxirane is protonated by the acid catalytic group, then the nucleophilic carboxylate forms an ester bond with the activated oxirane, giving a covalent-inhibitor complex. The high potency of cyclophellitol among the six inhibitors may be due to facile trans-diaxial opening of the epoxide ring at C-1 which follows the Fürst and Plattner rule.[19] The (*2S*)-diastereoisomer and 6-deoxy-1,2-epoxy analogue 1 which possess ß-epoxide are also expected to undergo trans-diaxial oxirane opening. This process is energetically favourable. Comparatively, the ring opening at C-1 for α-epoxide compounds, (*1R,6S*)-, (*1R,2S,6S*)-diastereoisomers and 6-deoxy-1,2-epoxy analogue 2 is energetically unfavourable because of the diequatorial opening. It is thus understandable why the inhibitors reacts less effectiveness with α-glycosidases.

Structurally, 6-deoxy-1,2 epoxy analogues 1 and 2 have the oxirane attached between C-1,2 instead of C-1,6 as in cyclophellitol. The IC_{50} of 6-deoxy-1,2 epoxy analogue 1 on ß-D-glucosidase was increased to 100 folds

compared with cyclophellitol. The relatively weak activity suggests that the position of epoxide ring is very important; this may be attributed to the fact that the oxygen atom of the epoxide ring is not at close proximity to the amino acid of the glycosidase. It is noteworthy that the oxygen atom of the oxirane in cyclophellitol has more or less the same orientation as the glucopyranosyl ring oxygen (O-5), thus allowing the protonation of the epoxide by the active site acid catalytic group. Another possible reason for the relatively weak activity of 6-deoxy-1,2 analogue 1 is the significance of the C-2 hydroxy group in the recognition of the specific enzyme. It is not surprising that 6-deoxy-1,2 analogue 1 is an inhibitor for both ß-D-glucosidase and ß-D-mannosidase as it contains the minimal structural features necessary for inhibition of both enzymes. The above reasons in complement with energetically unfavourable in opening of epoxide ring explains 6-deoxy-1,2-epoxy analogue 2 was inactive towards all the enzyme, particularly α-D-glycosidases.

Regarding the inhibition of unnatural (2*S*)-diastereoisomer on only fungal ß-D-mannosidase but not the snail enzymes, the considerable interest on the narrow specificity of the inhibitor on the target enzyme was aroused. The reason proposed is based on the finding of cyclophellitol. The presence of such pseudo-sugar, cyclophellitol in fungi presumably serves as a defensive role[2]. It may act as digestive glycosidase inhibitors to deter the invading or feeding of some lower class organisms such as other fungi, insect etc.

The inactivation mechanism of cyclophellitol and its analogues was comprehended based on the detailed kinetic studies. These compounds were therefore renamed as inactivators since the structure of an inactivator is altered by reaction with the enzyme (i.e. opening of epoxide). The term inactivator is used to distinguish these compounds from inhibitors, in which some of them do not have to covalently react with the enzyme during inhibition.

All compounds tested were found to be a highly specific irreversible inactivators of glycosidases. They appear to bind to the active site of enzyme, forming reversible/non-covalent E-I complexes prior to covalent bond formation. The rate constant value ki, describing the second step of inactivation mechanism actually, represents the transformation rate of reversible E-I complex into inactive one. Large ki values indicate that, at best, only a small amount of reversible complex are present. Once formed, these reversible complexes are rapidly converted into inactivated complexes into a faster rate. On the other hand, small ki value means the reversible complexes are present in certain amount due to the slow transformation rate.[28]

Ki (equilibrium constant of E-I complex) represents the affinity of E and I in the first step of reaction. Stronger inhibitors possess large ki values and small Ki values.

Judging from the ratio of ki/Ki, the effectiveness of each inactivator on the same enzyme could be revealed.[59] The data of two known irreversible

inactivators, conduritol aziridine and conduritol epoxide are also included in the following table for comparison.[6,32]

Table III.5. The kinetic constants for the inactivation of some glycosidases by cyclophellitol and its analogues, conduritol aziridine and conduritol epoxide.

Compound	Enzyme inhibited	Ki (mM)	ki (min^{-1})	ki/Ki (mM^{-1} min^{-1})	ki/Ki conduritol aziridine	ki/Ki conduritol epoxide
Cyclophellitol	ß-D-glucosidase (*Agrobacter sp.*)	0.055	1.26	22.9	0.026	-
	almonds	0.340	2.38	7.00	-	0.031[a]
(*1R,6S*)-diastereoisomer	α-D-glucosidase (Yeast)	0.026	0.401	15.4	0.041[b]	6.4 [c]
(*1R,2S,6S*)-diastereoisomer	α-D-mannosidase (Jack beans)	0.120	2.85	23.8	-	8.0×10^{-4}

[a] Enzyme source is sweet almond B. [b] α-glucosidase used is Type III from yeast whereas those used in my assay is type IV (both were obtained from Sigma company) [c] Yeast (*S. cerevisieae*), no type indication.

Cyclophellitol is indeed considerably more effective as an inactivator than conduritol aziridine (880 folds) and conduritol epoxide (226 folds) based on the comparison of ki/Ki ratios. Another two unnatural diastereoisomers were also more potent in inhibiting the enzyme with large ki/Ki values than the two conduritol compounds. Conduritol epoxide and conduritol aziridine lack a C-5 hydroxymethyl group in the structure, thus they are analogues of xylose, not glucose. Contribution of the hydroxymethyl group in cyclophellitol and its analogues makes them become a new class of potent glycosidase inhibitors.[59]

III.1.6 Further studies

Some glycosidase inhibitors such as deoxynojirimycin and castanospermine can inhibit the cytostatic effect of HIV by perturbing its gp120 glycosylation.[21] However, the cyclophellitol was not observed to have anti-HIV activity on infected CEM cell line (National Cancer Institute Development Therapeutics Program *in vitro* testing results) and on MT-4 cells.[2] It is proposed that the anti-HIV activity may be due to their ability to inhibit α-glucosidase but not ß-glucosidase. Some synthetic analogues in this project such as (*1R, 6S*)-diastereoisomer and (*1R, 2S, 6S*)-diastereoisomer are α-D-glycosidase inhibitors. It is valuable to screen the anti-tumor activity of whole series of compounds. In addition, specific inhibition nature of compounds suggested the assay should be carried out by using different types of infected cell lines.

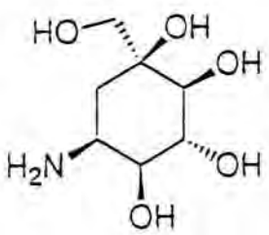
Up to now, the inactivation mechanism of cyclophellitol-like compound on glycosidase is clearly understood. Crystallographic identification of the enzyme-inhibitor complex and the ring opened product are encouraged to provide the additional information on the mechanism.

III.2 Reversible Competitive Inhibitors (Aminocyclitols)

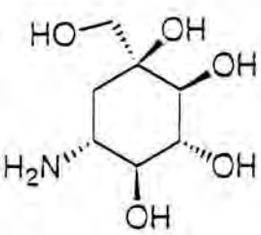
III.2.1 Introduction

As mentioned in section II.5.3, valiolamine (1S,2S,3R,4S,5S-1-amino-5-hydroxymethyl-cyclohexane-2,3,4,5-tetraol) was the most potent inhibitor among the 1-aminocarbasugars.[49] Chemical synthesis and modifications of this compound were done by L.-H. Wan at the Department of Chemistry. A series of compounds, namely valiolamine, its (*1R,2S*)-, (*1R,2R*)- and (*1S,2R*)-diastereoisomers were synthesised. In addition, an aminocarbasugar having an amino group at C-2 (2-amino-5-hydroxymethyl-cyclohexane-1,3,4,5-tetraol), a regioisomer of valiolamine, was also constructed. A total of five aminocyclitols were prepared.

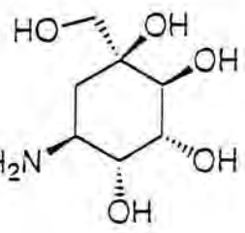
Fig. III. 8 Structure of valiolamine and its related aminocyclitols



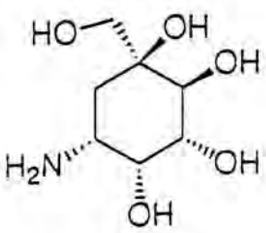
Valiolamine



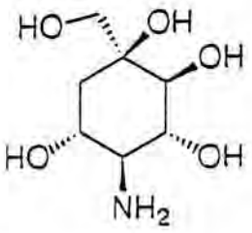
(*1R,2S*)-diastereoisomer



(*1S,2R*)-diastereoisomer



(*1R,2R*)-diastereoisomer



(*1R,2S,3R,4S,5S*)-2-amino-5-hydroxymethyl-cyclohexane-1,3,4,5-tetraol

All the five aminocyclitols have configurations similar with simple aldose sugars in which the ring oxygen was replaced by a methylene group and the hydroxy group at C-1 or C-2 was replaced by an amino group.

The amino group at C-1 is a crucial factor to make the inhibitor bind tightly to the enzyme and exert its inhibitory effect. During enzyme catalysis, there is an ion-pair formation between the carbonium ion at C-1 of the normal substrate and the carboxylate ion at the active site of the enzyme.[7,45] In aminocyclitols, the basic amino group at C-1 is also subjected to protonation which permits additional interactions with the binding site of the enzyme.[23]

In preliminary screenings, six commercial available glycosidases namely, α- and ß-D-glucosidases, α- and ß-D-mannosidases, and α- and ß-D-galactosidases were used instead of porcine intestinal sucrase, maltase and isomaltase[24]. Although the tested compounds might be less potent on these commercial available enzymes, its specific inhibition on certain enzyme should be revealed. IC_{50} and Lineweaver-Burk plot (L-B plot) were performed subsequently in order to establish the potency and mode of inhibition of each compound. The reversibility of these compounds were also investigated.

III-2.2 Materials

All the materials were purchased from Sigma Chemical Company.

Sugars: Maltose, 4-0-(α-D-glucopyranosyl)-D-glucose, approx. 98%, Cellobiose, 4-0-(ß-D-glucopyranosyl)-D-glucose.

Artificial substrates: All of the *p*-nitrophenyl glycosides listed in Table III.1.

Enzymes: (1) six glycosidases (see III.1.2); (2) glucose oxidase (EC 1.1.3.4), Type V-S: from *Aspergillus niger* (935 units/ml), solutions in 0.1 M sodium acetate buffer, pH approx.4 containing 0.002% thimerosal as preservative; (3) peroxidase (POD), donor: hydrogen peroxide oxidoreductase; (EC 1.11.1.7) from horseradish Type II, 175 purpurogallin units/mg solid. One unit will form 1.0 mg of purpurogallin from pyrogallol in 20 seconds as pH 6.0 at 20 °C.

Other reagents: *o*-dianisidine (3,3'-dimethoxybenezidine ; Fast Blue B) dihydrochloride, trizma-base(tris[hydroxymethyl] aminomethane) and Triton X-100 (Octyl Phenoxy Polyethoxyethanol)

Tris Glucose Oxidase Reagent (TGO)[10]: For the preparation of the TGO reagent, the following stock solution were used : (a) Tris buffer. Tris can

effectively inhibit the contaminant disaccharidases without interfering with the glucose oxidase method. A solution of 61.0 gm (0.5 mole) of Tris base in 85 ml of 5 N HCl was diluted to 1000 ml with distilled water. The pH of the solution was adjusted to 7.0. (b) glucose oxidase solution, 935 units/ml (c) peroxidase solution, a solution of peroxidase (175 purpurogallin units/ml), 1 mg/ml in distilled water which was kept at -20 °C. (d) *o*-dianisidine dihydrochloride solution (chromophore). A solution of *o*-dianisidine dihydrochloride, 10 mg/ ml in distilled water. This solution should be kept in dark. (e) detergent solution, a solution (10 ml) of Triton-X-100 in 40 ml of 95% solution which can help to keep the colored oxidation product of *o*-dianisidine in a clear solution. Triton X-100 which is free of peroxides, doesn't yield a brown colour with the TGO reagent and interfere with the enzymatic reaction.

Preparation of TGO reagent[10]: The glucose oxidase solution was first shaken with Tris buffer for half min and then the peroxidase solution, the *o*-dianisidine solution and detergent solution were added. The reagent contained 5.10 units/ml of glucose oxidase and 0.9975 purpurogallin units/ml of peroxidase. It was stored in the dark at 4 °C and stable for only few days.

II.2.3 Methods

III.2.3.1 Assay of glucoside hydrolase inhibition activity

The method of Kameda *et al* was followed.[24] The inhibitory activity was determined by incubating a solution (0.25 ml) of glucose hydrolase (α- or ß-D-glucosidase) in 0.02 M phosphate buffer with a 0.2 M substrate solution (0.25 ml) and 0.5 ml of inhibitor (different concentrations) at 37 °C for 9 min (ß-D-glucosidase) or 10 min (α-D-glucosidase). The incubation times chosen were based on the linear absorbance changes within the assay period and absorbance values less than 1.0 were obtained in this period. The enzyme reaction was quenched by the immersion of the tube in boiling water for 3 min. After cooling, the amount of released product (D-glucose) was determined using the glucose oxidase method described below.[10] All trials were in duplicate.

III.2.3.2 Glucose oxidase method for determination of released D-glucose[10]

For the determination of the glucose , 0.5 ml of the reaction mixture was transferred to another test tube. At the same time, a standard series containing 0, 0.1. 0.3, 0.5 ml of glucose solution (0.1 mg/ml) in distilled water were prepared to give a combined volume of 0.5 ml. At this point, 3 ml of TGO reagent was added to all tubes. The tubes were then incubated at 37°C for 30 min and then measured spectrophotometrically at 420 nm.

With certain substrates, maltose and cellobiose, the contaminant

enzymes (e.g. maltase) in TGO reagent could hydrolyse them into D-glucose which caused the measured absorbance values to be higher. Thus, the tubes with only substrate solution without any components were needed for running the same assay. After 10 mins, 0.5 ml of solution was pipetted out for determining the amount of glucose which was released from the substrate by only the contaminated enzymes.

The actual absorbance value = measured Abs.(control,test sample)- Abs.(substrate effect)

III.2.3.3 Inhibitory assay of aminocyclitol on other glycosidases[25]

The assay details were similar to those described in III.1.3.1 with slight differences. The reaction mixture, 0.25 ml of glycosidase (α-, ß-D-mannosidase ; α-, ß-D-galactosidase) dissolved in 0.02 M appropriate buffer, 0.25 ml of 0.01 M *p*-nitrophenyl glycoside and 0.5 ml of inhibitor solution (various concentration) was incubated at 30°C for about 10 mins. The residual enzyme activity was determined by colorimetric method. "Control" was included by replacing the inhibitor solution with distilled water.

Table III.6 The conditions (enzyme units, substrate, pH and the buffer used) for inhibitory assay of aminocyclitols on glycosidases

Enzyme	Final enzyme unit (mU/ml)	Substrate	Buffer (pH)
α-D-glucosidase (brewers yeast)	312.5	Maltose	Phosphate (pH=6.8)
ß-D-glucosidase (almonds)	149.7	Cellobiose	Sodium acetate (pH=5.0)
α-D-mannosidase (jack beans)	50.0	*p*-nitrophenyl α-D-mannopyranoside	Sodium acetate (pH=4.5)
ß-D-mannosidase (snail acetone powder)	50.0	*p*-nitrophenyl ß-D-mannopyranoside	Sodium acetate (pH=4.0)
ß-D-mannosidase (partially purified from *A. oryzae*)	27.3		
α-D-galactosidase (*E. coli*)	50.0	*p*-nitrophenyl α-D-galactopyranoside	Phosphate (pH=6.5)
ß-D-galactosidase (*A. oryzae*)	50.0	*p*-nitrophenyl ß-D-galactopyranoside	Sodium acetate (pH=4.5)

III.2.3.4 Influence of dialysis on the glycosidase inhibition[2]

A solution of α-D-glucosidase (final enzyme concentration, 312.5 mU/ml) was incubated with different concentration of inhibitors at 37 °C for 1 hour. One half volume was dialysed against the 10 mM of phosphate buffer at 4 °C for 12 hours. The remaining solution was stood at the same temperature and for the same time without dialysis. The control and dialysed samples were then pipetted out to determine the enzyme activity. The procedures given in III.2.3.1 was followed.

For α-D-mannosidase, 50 mU/ml of enzyme was incubated with different concentration of inhibitors. The incubation time was 1 hour at 30 °C. The dialysis procedures was carried out as before. The residual enzyme activity was assayed by mixing 0.375 ml of dialysed or control samples with 0.125 ml of 0.01 M *p*-nitrophenyl-α-D-mannopyranoside and incubated at 30 °C for 9 min. (same as III.2.3.3)

III.2.3.5 Lineweaver-Burk plot[24,25]

The activities of enzyme alone on various concentrations of the substrate were assayed as follows: 0.25 ml of 1.25 U/ml α-D-glucosidase in 0.02 M phosphate buffer, 0.25 ml of maltose (0.01, 0.014, 0.02, 0.04 and 0.08 M) and 0.5 ml of H_2O. The effects of the inhibitor on the enzyme activity were also determined using different concentrations of maltose as listed before. At least two concentrations of inhibitors were tested to construct the lines. All the test tube were incubated at 37°C for 10 min. The amount of D-glucose released was also measured by glucose oxidase method (refer to III.2.3.2).

For α-D-mannosidase, a similar assay method mentioned above was used. The reaction mixture contained same components but the substrate concentration was varied (1.25, 2.5, 3.75, 6.25, 7.5 mM). One set of trials was enzyme alone. The other three sets were three different concentration of inhibitors.

III.2.4 Results

III.2.4.1 Inhibitory activities of valiolamine and related aminocyclitols against glycosidases

The inhibitory activities of valiolamine and related aminocyclitols have been investigated and the results are shown in Table III.7.

Table III.7 Inhibitory activities of valiolamine and its related aminocyclitols against six glycosidases in the preliminary screenings.

Enzyme	% Inhibition				
	Valiolamine	(1R,2S)-diastereoisomer	(1S,2R)-diastereoisomer	(1R,2R)-diastereoisomer	Regioisomer[f]
α-D-glucosidase (brewers yeast)	92[a]	88[b]	28[c]	32[d]	2.1[e]
β-D-glucosidase (almonds)	43	12	5.8	5.8	11
α-D-mannosidase (jack beans)	20	38	85	6.6	1.9
β-D-mannosidase (snail acetone powder)	3.6	8.3	6.0	0	0
α-D-galactosidase (*E. coli*)	31	0.54	0	0	ND
β-D-galactosidase (*A. oryzae*)	95	60	61	82	ND

[a]Inhibition at the final concentration of 3.22 mM. [b]Inhibition at the final concentration of 2.89 mM. [c]Inhibition at the final concentration of 4.40 mM. [d]Inhibition at the final concentration of 4.43 mM. [e]Inhibition at the final concentration of 3.23 mM. [f]Regioisomer of valiolamine: 2-amino-5-hydroxymethyl-cyclohexane-1,3,4,5-tetraol. ND: not determined

All the aminocyclitols were synthesized chemically. The above table (Table III.7) shows the percentage inhibition on each enzyme at a certain concentration of aminocyclitol which is indicated in the legend. In order to compare the inhibitory effects of compounds, the concentrations used were around 3-4 mM in the preliminary screenings. Too small a concentration might conceal the inhibition effect of the compound whereas too large would give false results due to the saturation effects (vide infra). The concentrations used (in mM range) were according to those reported.[24] For those compounds which showed strong inhibition of the glycosidases with IC_{50} greater than 10^{-3} mM are summarised in Table III.8.

Table III.8 The enzyme specificity and IC_{50} of valiolamine and its related aminocyclitols

Inhibitors	Enzyme inhibited	IC_{50}
Valiolamine	α-D-glucosidase (brewers yeast)	4.50×10^{-4} M
	ß-D-galactosidase (*A. oryzae*)	1.90×10^{-3} M
	ß-D-glucosidase (almonds)	3.50×10^{-3} M
	α-D-galactosidase (*E. coli*)	5.14×10^{-3} M
(*1R,2S*)-diastereoisomer	α-D-glucosidase (brewers yeast)	5.00×10^{-4} M
	ß-D-glucosidase (almonds)	3.29×10^{-3} M
	ß-D-galactosidase (*A. oryzae*)	3.45×10^{-3} M
(*1S,2R*)-diastereoisomer	α-D-mannosidase (jack beans)	1.95×10^{-3} M
	ß-D-galactosidase (*A. oryzae*)	3.86×10^{-3} M
(*1R,2R*)-diastereoisomer	ß-D-galactosidase (*A. oryzae*)	3.50×10^{-3} M
Regioisomer	-	-

As shown in Table III.8, each compound inhibited more than one enzyme. The aminocyclitols did not appear to be specific enzyme inhibitors, unlike the cyclophellitol mentioned before (III.1).

Valiolamine, a aminocyclitol readily isolated from the fermentation broth of *Streptomyces hygroscopicus*, subsp. *limoneus* was shown to inhibit yeast α-glucosidase, *A. oryzae* ß-galactosidase, almonds ß-glucosidase and *E. coli* α-galactosidase in order of effectiveness (shown in Table III.7). The IC_{50} values were consistent with those reported by Kameda *et al* in 1984 with the same order of magnitude (IC_{50} values of α-glucosidase and almonds ß-glucosidase were 1.9×10^{-4} and 8.1×10^{-3} M by Kameda *et al.*)[24] Kameda *et al* also examined the effect of valiolamine on porcine intestinal maltase, sucrase, isomaltase, sucrase, glucoamylase, α- and ß-amylase.[24] The compound was considerably more active against the former three than the enzymes listed in Table III.7. Owing to the inaccessibility of enzyme source, only the common glycoside hydrolases were screened in this study.

The results between valiolamine and its (*1R,2S*)-diastereoisomer were compared and showed that both were relatively potent inhibitors of yeast α-glucosidase, regardless of the configuration of the amino group at C-1. It was originally expected that (*1R,2S*)-diastereoisomer should be an inhibitor of ß-glucosidase due to its ß-amino group. However, the IC_{50} on ß-glucosidase was

even greater than α-glucosidase. This result was totally contrary to the initial expectation. In addition, the inhibition patterns of the two compounds on ß-glycosidase were quite strange.

Fig. III.9 shows the inhibition of (*1R,2S*)-diastereoisomer on almonds ß-D-glucosidase. At low concentration, there was no or little inhibition on the enzyme. However, when the concentration increased, there was a sharp rise in percentage inhibition at narrow range of concentrations (see discussion section).

Moreover, it was interesting to note that almost all the compounds could inhibit ß-galactosidase. The reason for the wide susceptibility of this enzyme was not clear. This may be related to the flexibility of the active site of the enzyme which can accommodate a number of compounds.

(*1S, 2R*)-Diastereoisomer was a unique enzyme which had remarkable inhibitory effect on α-D-mannosidase with $IC_{50}=1.96 \times 10^{-3}$ M. ß-Hydroxy group at C-2 and α-amino group at C-1 contribute its structure similarity to α-D-mannose. It inhibited the configurationally related enzyme. Conversely, (*1R, 2R*)-diastereoisomer which possess ß-amino group was expected to inhibit ß-D-mannosidase. Surprisingly, no inhibition was observed on ß-D-mannosidase (snail acetone powder) even applying 4.43 mM inhibitor,

(*1R,2R*)-diastereoisomer into the reaction mixture. Same circumstances had occurred in testing cyclophellitol and its analogues on the same enzyme (III.1.4.1). This might be attributed to the source of enzyme used. Another source, ß-D-mannosidase (*A. oryzae*) was used to test the compound again. The insufficient amount of partially purified ß-D-mannosidase only allowed the screening of three possible compounds including, (*1R, 2S*)-, (*1S,2R*)- and (*1R,2R*)-diastereoisomers. The results indicated that only (*1S,2R*)-diastereoisomer had effect against the fungal ß-D-mannosidase (15 % inhibition at 5.46 mM and 92 % at 17.06 mM) but not the others. (*1S,2R*)-Diastereoisomer seemed to be an inhibitor of both α and ß-D-mannosidase. This implied that the amino group configuration was not important in determining the configuration of enzyme inhibited.

In summary, (*1R,2R*)-diastereoisomer only had inhibition on ß-D-galactosidase. Because of the wide susceptibility nature of this enzyme, it was advisable not to characterize (*1R,2R*)-diastereoisomer as an inhibitor. The regioisomer of valiolamine (2-amino-5-hydroxymethyl-cyclohexane-1,3,4,5-tetraol) exhibited no inhibition on four enzymes listed in Table III.7. No inhibition assays were carried out on two galactosidases due to the insufficient amount of this synthetic compound.

III.2.4.2 Characterization the aminocyclitols as reversible competitive inhibitors

Similar to the previous studies, dialysis experiments were performed to reveal the nature of binding of each compound to the enzyme. Only the compounds which demonstrated the potent inhibition on the enzymes were tested. These included valiolamine, the (*1R,2S*)-diastereoisomer on α-glucosidase (Fig. III.10 & 11), and the (*1S,2R*)-diastereoisomer on α-mannosidase (Fig. III.12). In all cases, after dialysis, total enzyme activity was totally recovered and in some cases, recovered activity was even greater than the control sample (without inhibitor). This result may be attributable to the protection of the active site by binding of these reversible inhibitors. Denaturation of enzyme would be lessened during a long period of dialysis . Irreversible binding due to covalent interaction was not easily released by water like this. As concluded, the binding was proved to be reversible based on the above evidences.

The competitive inhibition of α-glucosidase by both valiolamine and valienamine were illustrated by Lineweaver-Burk plot (mentioned in II.5.3). Measurements of the rate of catalysis at different concentration of substrate and inhibitors can serve to distinguish the mode of inhibition action on the enzyme. The plot is generally applied in studying reversible types of inhibitor. In competitive inhibition, the y-intercept of the plot 1/V versus 1/S is the same in the presence or absence of inhibitor but the slope is different. In non-

competitive inhibition, the y-intercept ($1/V_{max}$) is increased but the slope is unchanged. Both two parameters (y-intercept and slope) are changed in uncompetitive inhibition.[9]

As shown by the L-B plot in Fig. III.13, valiolamine was competitive with maltose and such a relationship had been seen previously in using intestinal α-glucosidase (Fig. II.12). At a sufficiently high substrate concentration, virtually all the active sites were filled by substrate and the enzyme was fully operative. And V_{max} was not altered by the inhibition shown in Fig. III.13. Same phenomenon was observed in both (*1R,2S*)-diastereoisomer on yeast α-D-glucosidase (Fig. III.14) and (*1S,2R*)-diastereoisomer on jack beans α-D-mannosidase (Fig. III.15). All of the plots had the same y-intercept but had different slopes. The Ki values of valiolamine and (*1R,2S*)-diastereoisomer for brewers yeast α-D-glucosidase were found to be 1.2×10^{-4} M and 2.5×10^{-4} M respectively, which are 10^{-2} smaller than the Km values (4.4×10^{-2} M for maltose). From a comparison of Ki and Km values, it was concluded that these two inhibitors had strong affinity to the enzyme for forming EI complex than the substrate. The strong competitive inhibitions were shown. For inhibition of jack beans α-D-mannosidase by (*1S,2R*)-diastereoisomer, the Ki value was 2.3×10^{-3} M which is close to the Km value for *p*-nitrophenyl-mannopyranoside (3.1×10^{-3} M). The affinity to the enzyme for both inhibitor and substrate are similar in this case.

As mentioned before, the inhibition pattern of ß-glycosidase is abnormal (Fig. III.9). In addition, the competitive mode of the action seems to be valid at only low concentration of inhibitor in L-B plot. At high inhibitor concentration (3.00 mM), the line deviated above without overlapping of the y-intercept (Fig. III.16)

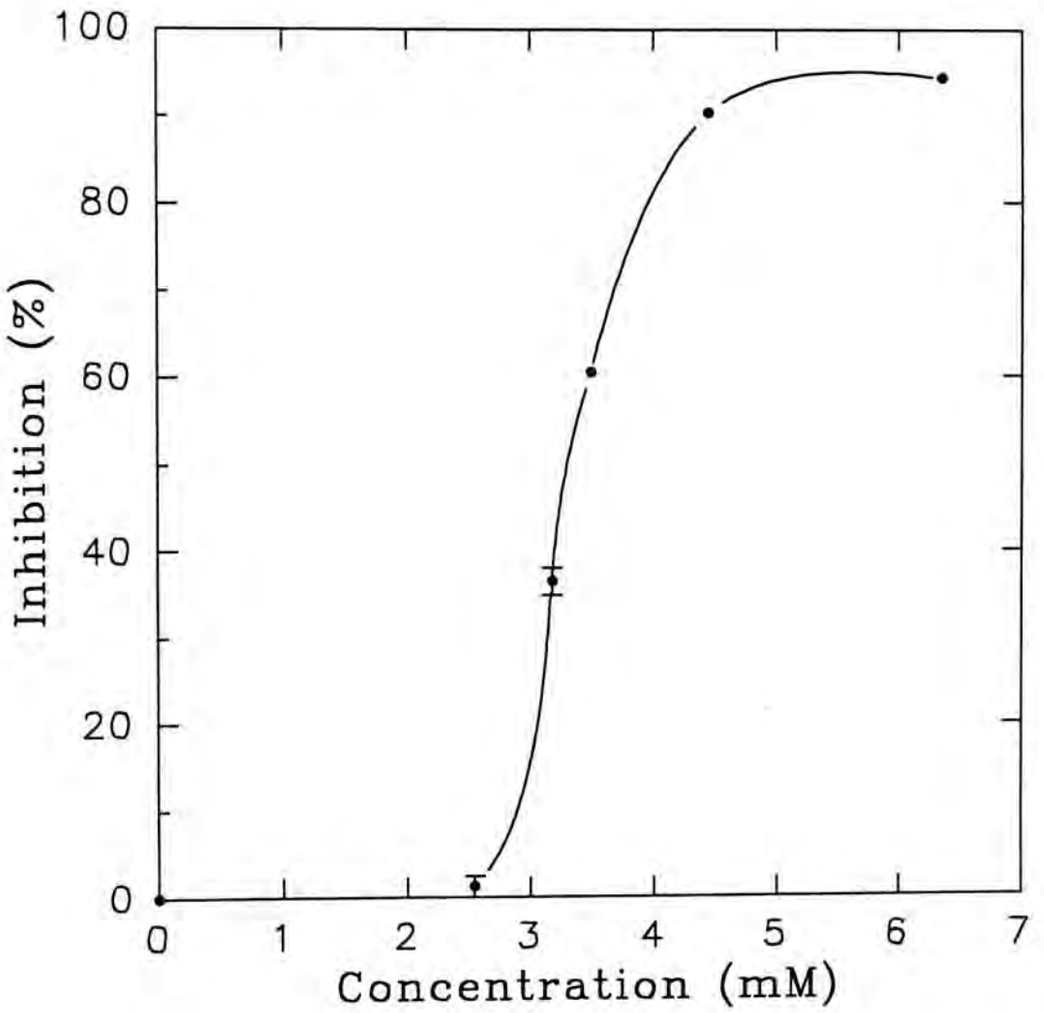


Fig. III.9 Inhibition of almonds β-D-glucosidase by (*1R,2S*)-diastereoisomer of valiolamine with enzyme conc. 149.7 mU/ml)

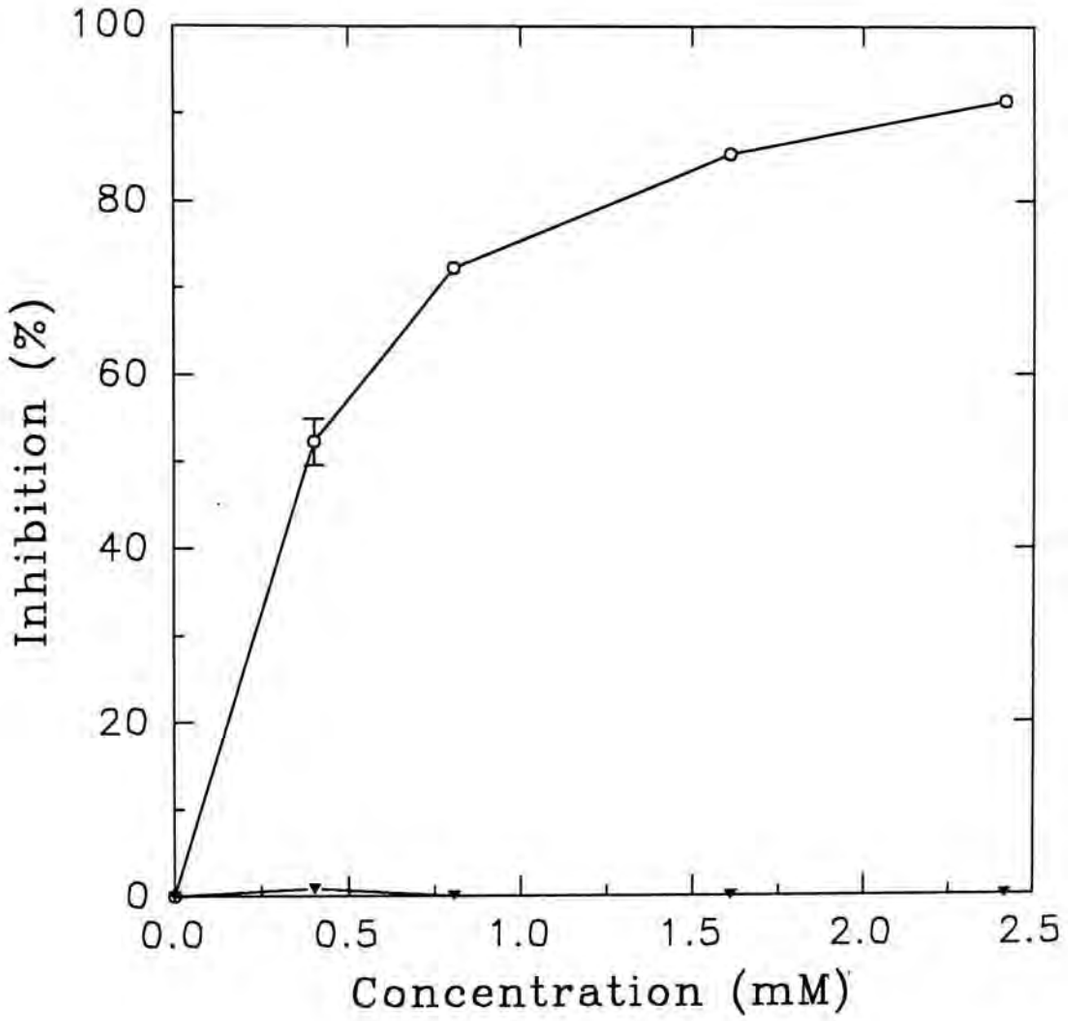


Fig. III.10 Effect of dialysis on the inhibition of brewers yeast α-D-glucosidase by valiolamine with enzyme conc. 312.5 mU/ml. One half volume was dialysed for 12 hr. at 4°C, dialysed sample (▼—▼). The rest was stood without dialysis, control sample (○—○).

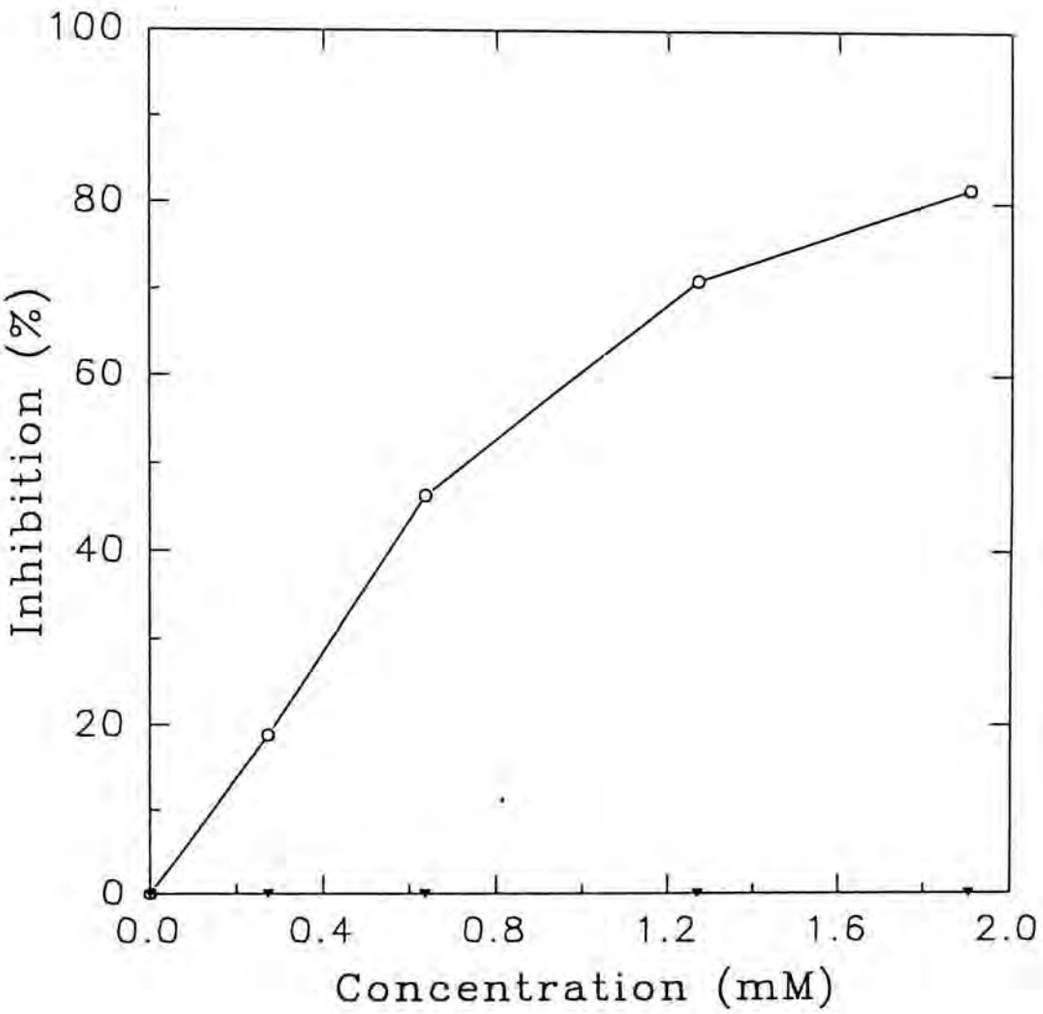


Fig. III.11 Effect of dialysis on the inhibition of brewers yeast α-D-glucosidase by (*1R,2S*)-diastereoisomer of valiolamine with enzyme conc. 312.5 mU/ml. One half volume was dialysed for 12 hr. at 4°C, dialysed sample (▼—▼). The rest was stood without dialysis, control sample (○—○).

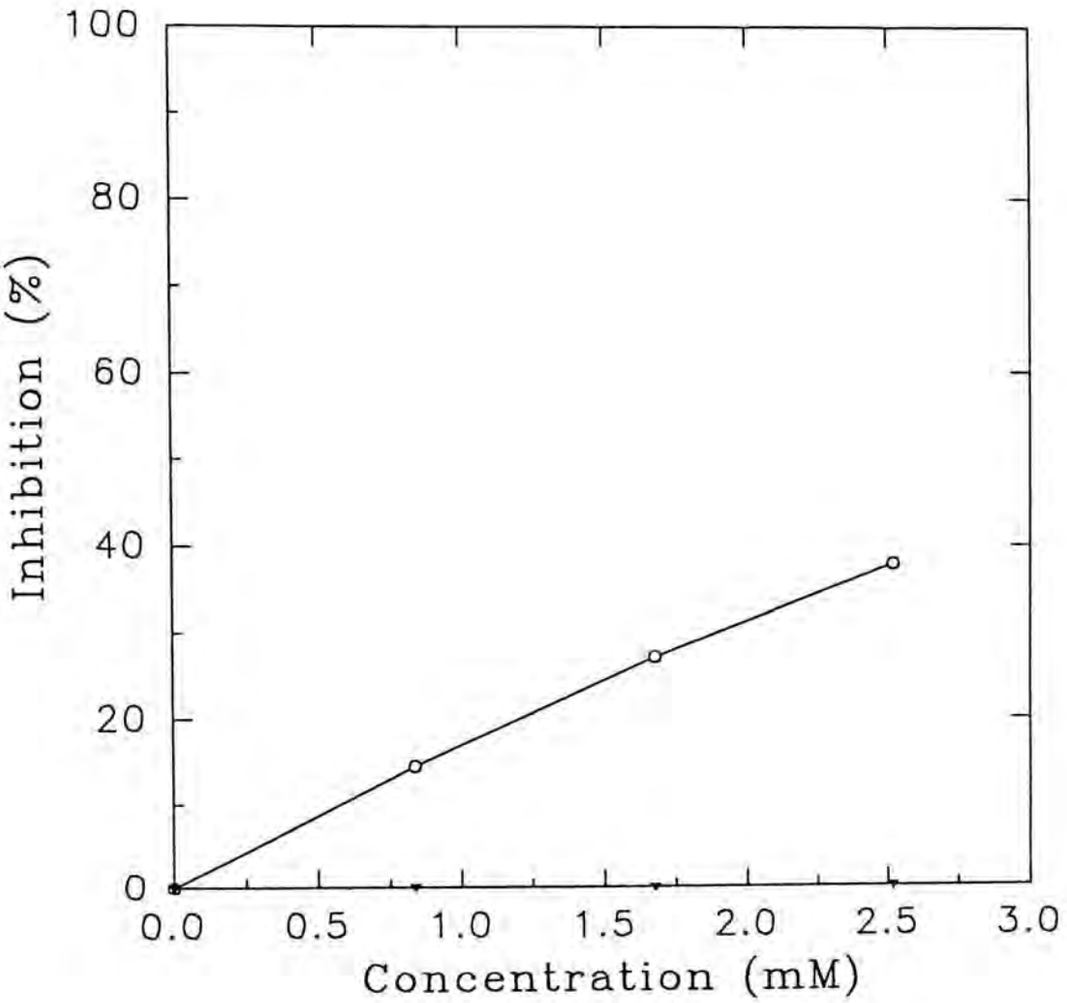


Fig. III.12 Effect of dialysis on the inhibitition of jack beans α-D-mannosidase by (*1S,2R*)-diastereoisomer of valiolamine with enzyme conc. 50 mU/ml. One half volume was dialysed for 12 hr. at 4°C, dialysed sample (▼—▼). The rest was stood without dialysis, control sample (○—○).

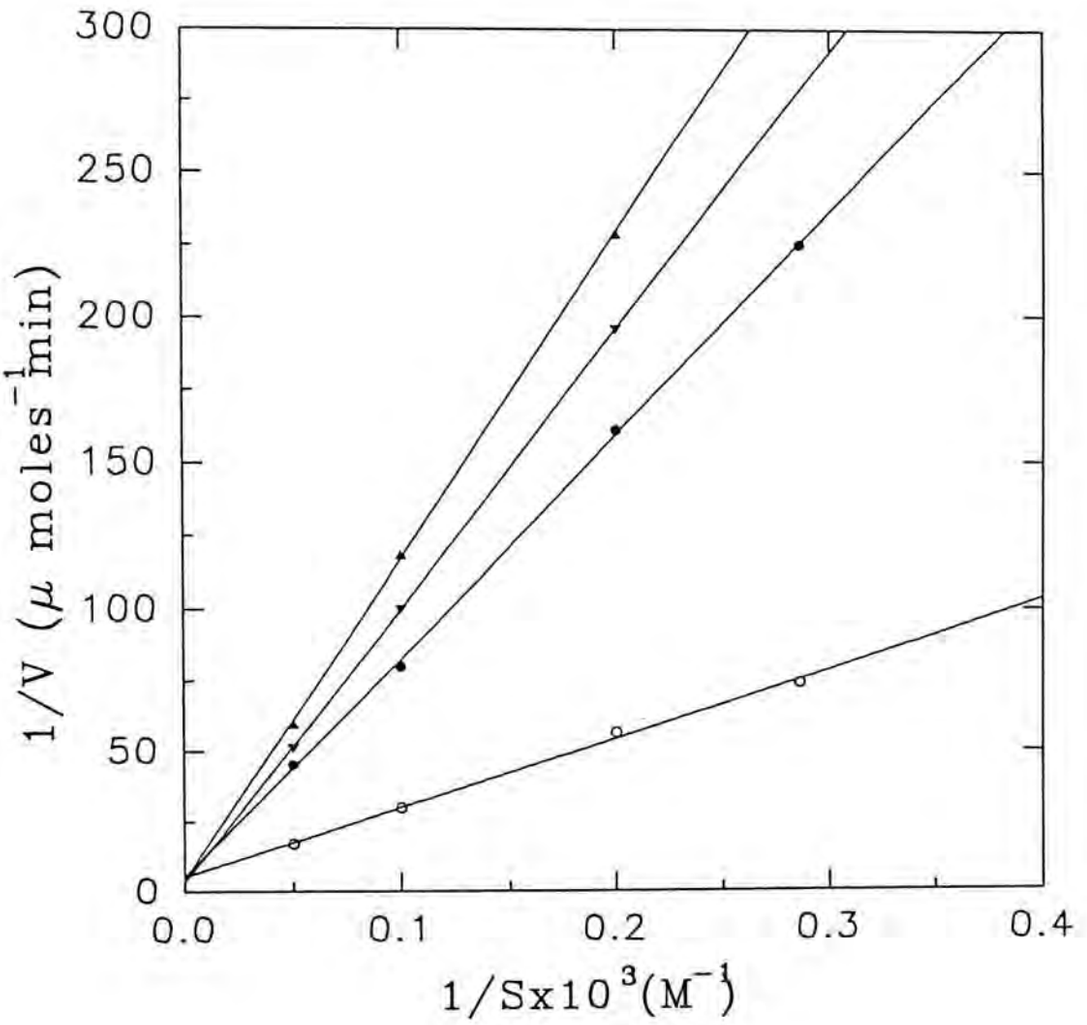


Fig. III.13 Effect of valiolamine on hydrolysis of maltose by brewers yeast α-D-glucosidase with enzyme conc. 312.5 mU/ml. Concentration of valiolamine employed: 0 mM, enzyme alone (○); 0.322 mM (●); 0.403 mM (▼); 0.483 mM (▲)

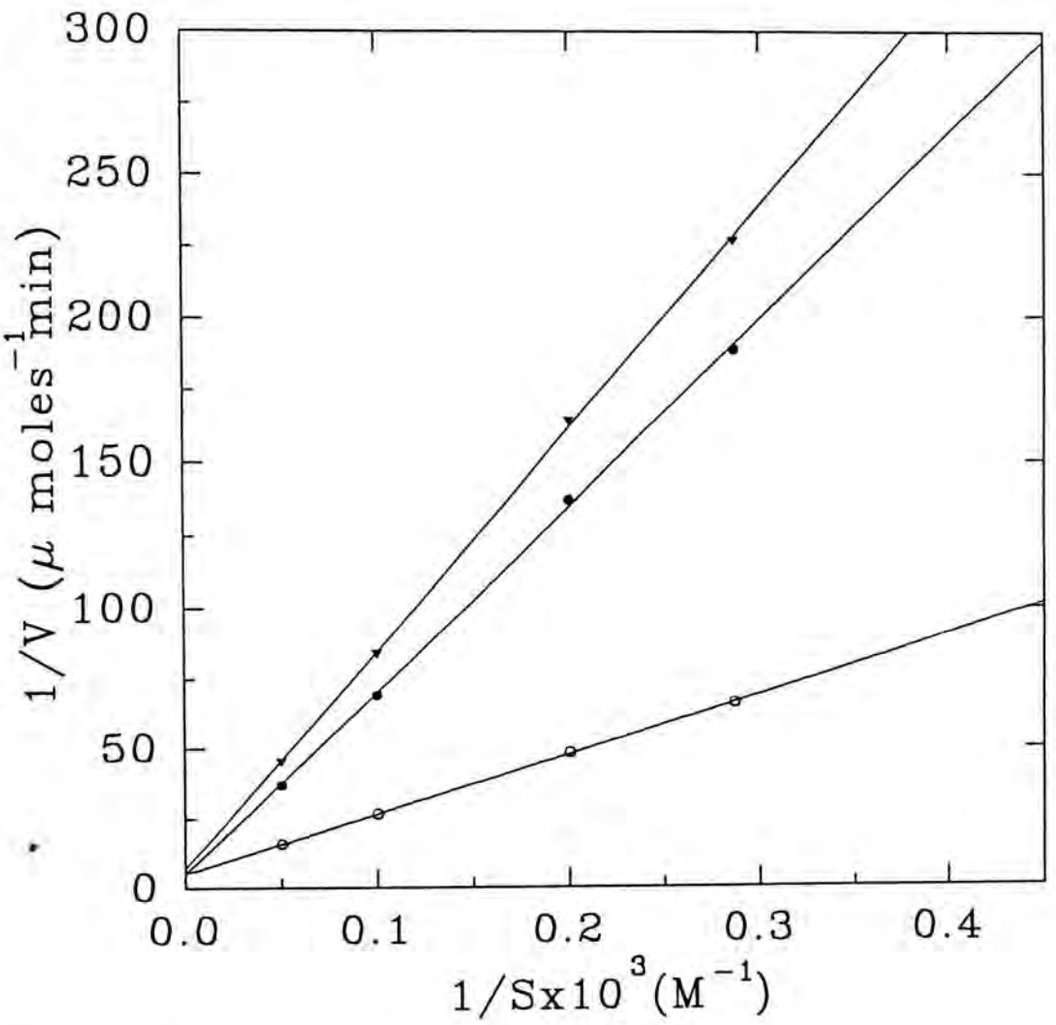


Fig. III.14 Effect of (*1R,2S*)-diastereoisomer of valiolamine on hydrolysis of maltose by brewers yeast α-D-glucosidase with enzyme conc. 312.5 mU/ml. Concentration of diastereoisomer employed: 0 mM, enzyme alone (○); 0.347 mM (●); 0.491 mM (▼)

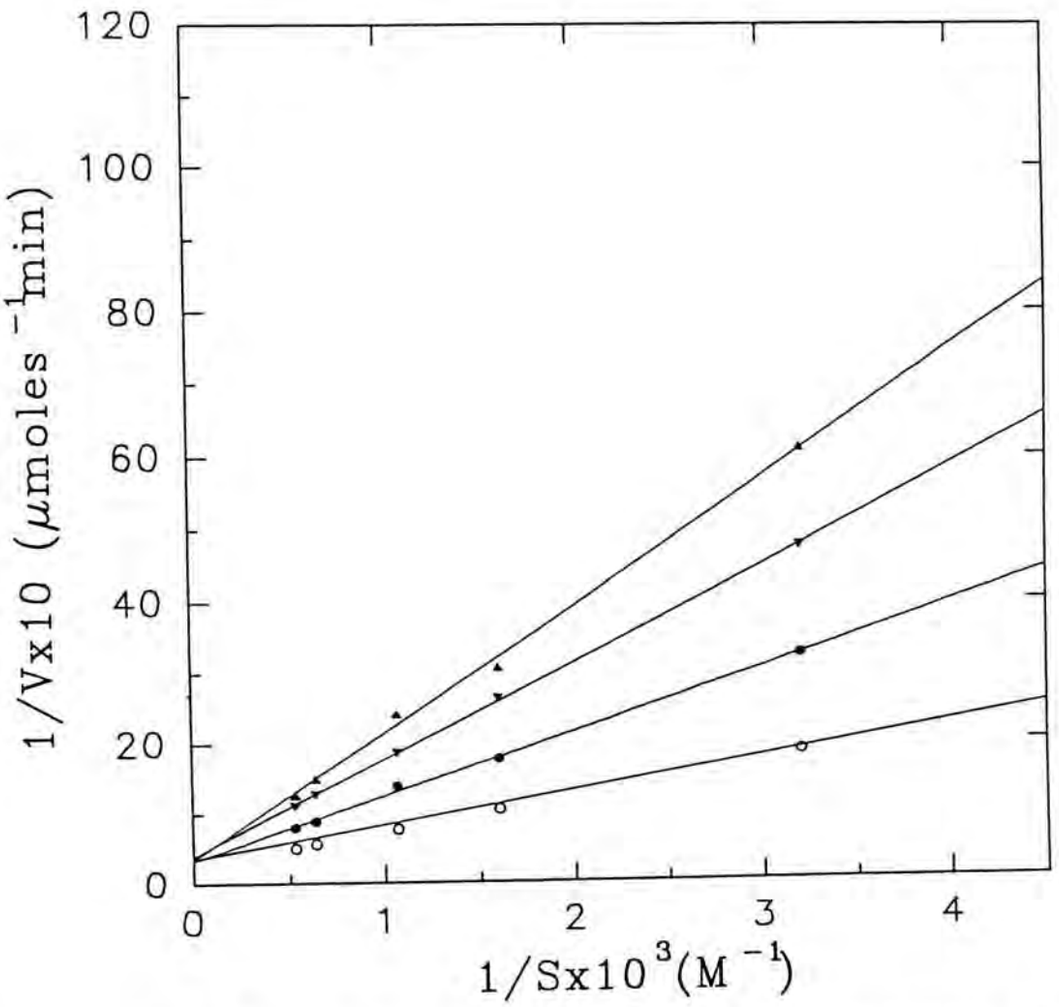


Fig. III.15 Effect of (*1S,2R*)-diastereoisomer of valiolamine on hydrolysis of *p*-nitrophenyl-α-D-mannoside by jack beans α-D-mannosidase with enzyme conc. 50 mU/ml. Concentration of diastereoisomer employed: 0 mM, enzyme alone (○); 0.919 mM (●); 1.38 mM (▼); 1.56 mM (▲)

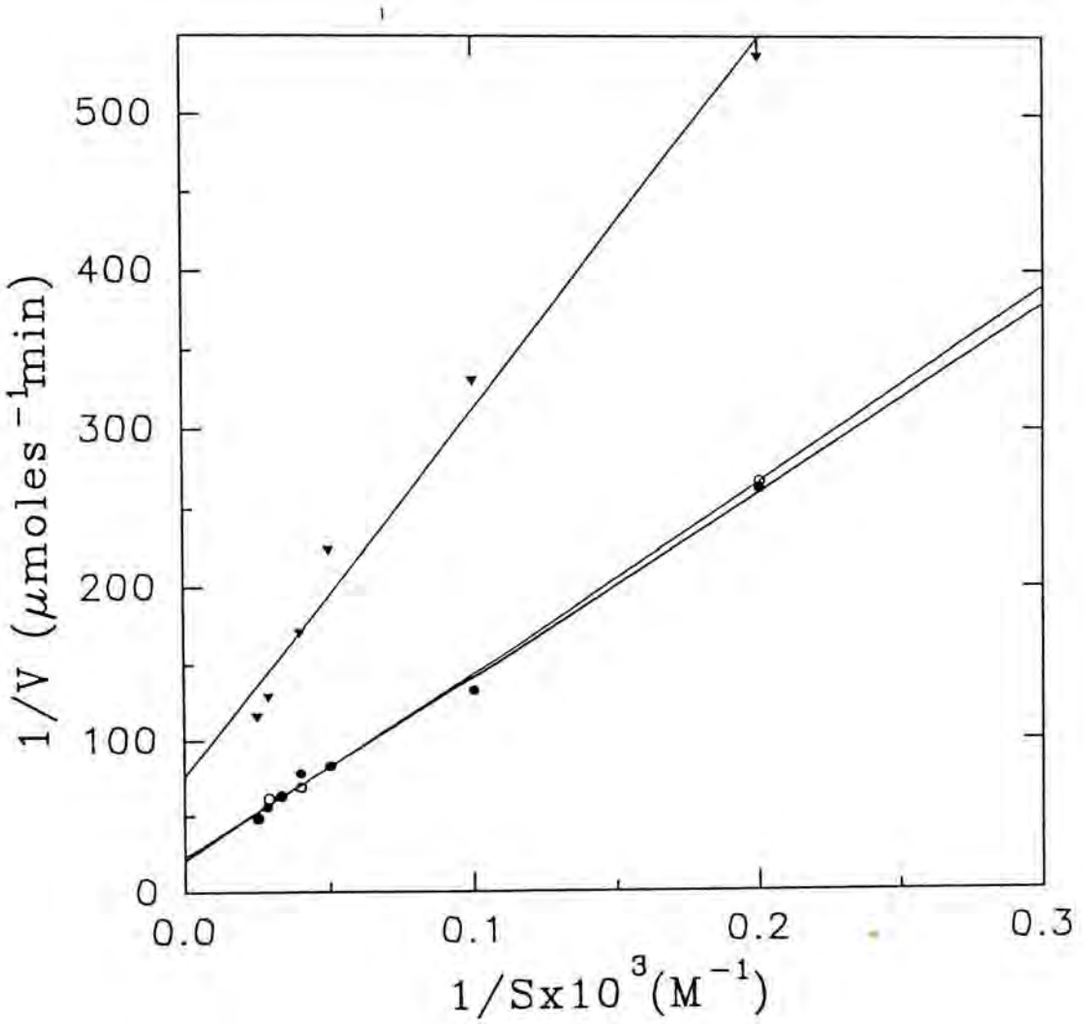


Fig. III.16 Effect of (*1R,2S*)-diastereoisomer of valiolamine on hydrolysis of cellobiose by almonds β-D-glucosidase with enzyme conc. 149.7 mU/ml. Concentration of diastereoisomer employed: 0 mM, enzyme alone (○); 2.15 mM (●); 3.00 mM (▼)

III.2.5 Discussion

In 1980, Kameda *et al* proposed that the inhibitory effect of one aminocyclitol, valienamine, was their structure similarity to the D-glycosyl cation forming a half-chair conformation on the transition state in the course of enzyme-catalysed pyranoside hydrolysis.[25] The flexibility of the cyclohexane ring enable aminocyclitols to achieve the half-chair conformation of the transition state and assist the binding during inhibition. In addition, the protonation of amino group in inhibitor presumably accounts for some of the inhibitory power.[23] It resembles the positive charge of carbonium ion in transition state and enhance the electrostatic binding to the enzyme.

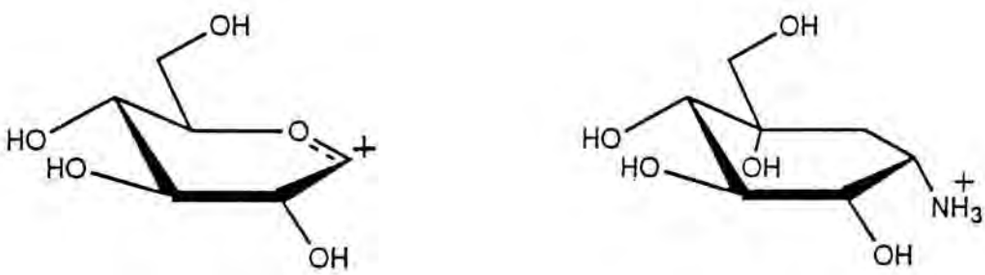


Fig. III.17 Half-chair conformation and positive charge make valiolamine resemble a glycosyl cation during enzyme inhibition

In glycosidase-catalysed hydrolysis, the anomeric oxygen at C-1 position is protonated by acid catalytic group leading to half chair carbonium ion like intermediate. The configuration of anomeric oxygen, i.e. the glycosidic linkage, is important in determining the specificity of the enzyme. It is expected that the enzyme inhibited should correspond to the configuration of amino group at C-1 of the inhibitor. However, both valiolamine and its (*1R,2S*)-diastereoisomer inhibited α-D-glucosidase whereas (*1S,2R*)- and (*1R,2R*)-diastereoisomer inhibited α-D-mannosidase, regardless of the amino group configuration at C-1. The possible reason is that the weak basic properties of these compounds affect the specific interaction to the active site of the enzyme. In addition, lack of pyranoid-ring oxygen or nitrogen may also affect the specificity of inhibitor towards the enzyme.

Shifting the amino group from the C-1 to the C-2 position especially abolishes the inhibitory effect of the compound 2-amino-5-hydroxyl-cyclohexane-1,3,4,5-tetraol. No protonation of the amino group was feasible due to the incompatible position between the amino group and acid catalytic group of the enzyme. The normal electrostatic interaction might therefore be strongly affected which would diminish the binding of compound to the enzyme and resulting in low inhibitory power.

In general, the aminocyclitols are less potent inhibitors for α- and ß-D-glucosidases than the compounds deoxnojirimycin and glucosylamine.

Although the replacement of the pyranoid-ring oxygen/nitrogen atom with a methylene group did not result in complete loss of inhibitory activity, it still affects the half chair geometry. Aminocyclitols with nitrogen not in ring structure have less stability in aqueous solution compared with those have imino group in the ring.[32] Both factors appear to affect the inhibitory potency of the aminocyclitols. In addition, less basic properties with little electrostatic interaction to the enzyme may also diminish its potency.

The abnormal inhibition properties of some aminocyclitols on ß-glycosidase may reflect denaturation effects. It is conceivable that the compound may inhibit the enzyme competitively at low concentration (shown by L-B plot, Fig. III.15). However, when the concentration increases, the abundant amount of inhibitor in solution may affect the ionic strength of the reaction mixture. The inhibitory effect at elevated concentration may be due to binding of the inhibitors to the active site and to denaturation of the enzyme. As a result, the percentage inhibition dramatically increased. Elevated concentrations of inhibitors may elicit this situation and give false results.

CHAPTER IV Isolation of the Naturally Occurring Glycosidase Inhibitors from Mushroom.

IV.1 Introduction

Cyclophellitol, a potent ß-D-glycosidase inhibitor, was isolated from the culture filtrate of the fungus *Phellinus* sp. in 1989.[3]

Four common edible mushrooms, *Lyophyllum aggregatum*; *Volvariella volvacea*; *Pleurotus sajor-caju* PL-27 and *Ganoderma lucidum* were screened for their inhibitory effects on six glycosidases namely as α-, ß-D-glucosidase, α-, ß-D-mannosidase, α-, ß-D-galactosidase by Tsang Y. L. and Y. S. Wong in 1992.[56] Their results showed that the water extract of *Ganoderma lucidum* had significant and specific inhibitory effects on both α- and ß-D-glucosidases, as well as α-D-galactosidase. Since *Phellinus* and *Ganoderma* belong to the same family, *Polyporaceae*, further studies on identifying putative glycosidase inhibitors in *Ganoderma lucidum* were carried out.

IV.2 Materials

Mushrooms: Dried slices of Ganoderma lucidum fruit body were imported from China, Flesh fruit bodies of *Volvariella volvaceae* were purchased from the local market.

Enzyme Assays: The substrates, *p*-nitrophenyl glycoside, maltose and methyl-α-D-glucopyranose were purchased from Sigma Chemical Company, as were the α-D-glucosidase (Type VI; from brewers yeast), ß-D-glucosidase (almonds), α-D-mannosidase (from jack beans), ß-D-mannosidase (from snail acetone powder), α-D-galactosidase (from *Escherichia coli*) and ß-D-galactosidase (from Aspergillus oryzae).

Dialysis: Molecular porous dialysis membrane (MWCO: <2,000) was purchased from Spectrum Company.

Reagents of anthrone method: Anthrone method and glucose standard solution (100mg/ml with 0.1% benzoic acid as preservative) were purchased from the Sigma Chemical Company. Thiourea, (A.R. grade) was purchased from Peking Chemical Works.

Liquid column chromatography: Flash liquid chromatography was carried out on columns of Merck Keisel gel 60 (70-230 mesh, E. Merck No. 9385). The fractions eluted were monitored by thin layer chromatography (TLC) on Merck precoated silica gel $60F_{254}$ plates. All solvents used were reagent grade.

Nuclear magnetic resonance and Mass spectrophotometry: ^1H NMR spectra were recorded with a Bruker WM250 at 250 MHZ and a Joel-270 spectrophotometer at 270 MHz for solution in D_2O (DOH = 4.80 ppm). The reference standards, adonitol and xylitol were purchased from Sigma company. ß-(L)-(+)-Arabitol was prepared by reacting ß-(L)-(+) arabinose with sodium borohydride (1:1, v/v) in methanol. (This part was kindly done by Mr. Vincent W.-F Tai). EI-CI mass spectra were taken by a HP5989A spectrophotometer using at 70 ev for electron impact method.

IV.3 Methods

IV.3.1 Preparation of *Ganoderma lucidum*

Ganoderma lucidum slices were cut into small pieces. One hundred gram of tissues was boiled in 1.5 litre water for 2 hour. The residue was further boiled in 1 litre water for 1 hour. The pooled aqueous fraction was filtered through Whatman #1 paper and lyophilized. This crude extract was redissolved in water (50 mg/ml) and dialysed against water in tubing with MW cutoff <2000 for 24 hour (crude extract 100 ml; water 3 litre). The solution outside the tubing was collected and lyophilized. This sample is designated as partially purified extract.

IV.3.2 Preparation of *Volvariella volvacea*

One hundred gram of fruit bodies of *V. volvacea* were homogenized in 150 ml water with a Waring blender at high speed for 2 min. The homogenate was filtered through 2 layers of cheese cloth to remove cell debris. The filtrate was boiled for 10 min and centrifuged at 27500 xg for 15 min. The supernatant was collected and lyophilized.

IV.3.3 Inhibitory assay of aqueous extract of mushrooms on glycosidases

Glycosidase assay was as described in section III.1.3.1. The reaction mixture contained 0.1 ml of 100 mM appropriate buffer, 0.2 ml of 12.5 mM *p*-nitrophenyl glycoside, 0.1 ml of glycosidase and 0.1 ml of mushroom extract in a final volume of 0.5 ml. Control was included by replacing the mushroom extract with water. Blank contained no enzyme and was run in parallel to account for the background colour of the mushroom extract. Reaction mixture was incubated at 30 °C for 10 min, 2.5 ml of 0.4 M glycine-NaOH solution was added to inactivate the enzyme and quench the reaction. Liberated *p*-nitrophenol was monitored at 410 nm. The net absorbance of tested sample was calculated as: $Abs._{(test)} = Abs._{(measured)} - Abs._{(blank)}$. And the results were presented as percentage inhibition on the enzyme, according the following formula:

$$\% \text{ inhibition} = \frac{Abs_{(control)} - Abs_{(test)}}{Abs_{(control)}} \times 100\%$$

IV.3.4 Anthrone method for determination of reducing sugars[38]

Anthrone reagent was prepared by adding 500 mg anthrone, 10 gm of thiourea in 1 litre of concentrated H_2SO_4 (66%). The mixture was warmed to 80-90°C with occasional shaking. After cooling, it was kept at 4 °C This reagent should be freshly prepared every week.

Ten ml of anthrone reagent was added to 1 ml sample solution in a

stoppered tube. The mixture was boiled for 15 min. After cooling for 30 min, the absorbance at 620 nm was measured. Water was used as blank. Glucose was used to prepare a standard curve. The amount of sugar was expressed as μg reducing sugar per mg of extract. No deproteinization was required for the *G. lucidum* extract which had been pre-dialysed (see IV.3.1)

IV.3.5 Flash liquid chromatography for purification of putative inhibitors in *G. lucidum* extract [47]

The partially purified extract of *G. lucidum* was extracted by methanol prior to chromatographic separation. The partially purified extract of *G. lucidum* were extracted in methanol (3 g in 100 ml) and heated at 50 °C for 1 hour. After removal of the residues by filtration, the methanol extract was concentrated and dried by evaporation and lyophilization. The extract powder (2 g) was preadsorbed to silica gel (5.5 g, 70-230 mesh, E. Merck No. 9385) and then evaporated to dryness *in vacuo* to give crude material.

The crude material (3.1 g) was then flash chromatographed on a column (2.5x18 cm) of silica gel (65 g) with 250 ml of $CHCl_3$: MeOH (5:1, v/v). The column was first eluted with 500 ml of $CHCl_3$: MeOH (5:1, v/v) and fractions of 75 ml were collected. The column was then eluted stepwise with the following solvents systems: 500 ml of $CHCl_3$: MeOH (4:1, v/v); 1.1 litre of 3:1 $CHCl_3$: MeOH and 200 ml of $CHCl_3$: MeOH (1:1, v/v). Fractions of 50 ml were collected in the subsequent elution. Aliquots of 5-10 μl from

fraction was spotted onto TLC plate and developed in the corresponding solvent system. The TLC plate was sprayed with concentrated sulphuric acid and heated to detect the presence of carbon-containing compounds which gave dark colour spots upon treatment. Each fraction was evaporated to dryness and redissolved in distilled water for glycosidase inhibition determination. Fractions with glycosidase inhibitory activities were pooled and lyophilized, and subject to second chromatographic purification.

For a typical second chromatographic run, the lyophilized sample obtained (0.4 g) was preadsorbed onto silica gel (0.7 g) and loaded onto a column (2x18 cm) of same silica gel as described previously. It was equilibrated with $CHCl_3$: MeOH (18:1, v/v) first and then eluted with a series of $CHCl_3$: MeOH solvent systems by increasing the methanol concentration, 170 ml (16:1, v/v), 75 ml (14:1, v/v), 100 ml (11.4:1, v/v), 180 ml (8:1, v/v), 70 ml (6:1, v/v), 120 ml (5:1, v/v), 160 ml (3:1, v/v), 90 ml (2:1, v/v) and 300 ml (1:1, v/v). Fraction size was 25 ml each. Fractions with α-D-glucosidase inhibitory activities were pooled.

Further purification was carried out by a third chromatographic run. Sample obtained from the second column (130 mg) was rechromatographed using a smaller column (1.3 x 1.3 cm) and eluted with $CHCl_3$: MeOH: NH_3 (45:40:15, v/v/v). Two compounds with α-D-glucosidase inhibitory activities were isolated. Structure of these two compounds were determined by nuclear magnetic resonance in D_2O solvent and mass spectrophotometry.

IV.4 Results

IV.4.1 Prescreening of Inhibitory Effects of Various Fungal Extracts

The inhibitory effects of crude and partially purified sample of four mushroom extracts on six glycosidases are tabulated in Table IV.1[56]

Table IV.1 The inhibitory effects of crude and partially purified sample of four mushroom extracts on six glycosidases

Enzyme	*Ganoderma lucidum*	*Lyophyllum aggregatum*	*Volvariella volvacea*	*Pleurotus sajor-caju*
	(a) (b)			
α-D-glucosidase	++ ++	- -	- -	- -
ß-D-glucosidase	++ ++	- -	- +	- +
α-D-mannosidase	- -	- -	+ +	+ -
ß-D-mannosidase	- -	- -	- -	- -
α-D-galactosidase	++ ++	- -	- -	- -
ß-D-galactosidase	- -	- -	- -	- -

(a): crude extract
(b): partially purified extract
+ - : degree of inhibition

Both crude and partially purified extract of *G. lucidum* specifically and significantly inhibited α- and ß-D-glucosidases and α-D-galactosidase. Partially purified sample exhibited stronger inhibition.

In this study, screening of glycosidase inhibitory activities of extracts of *G. lucidum* and *V. volvacea* was confirmed. Results were listed in Table IV.2

Table IV.2 The percentage inhibitions of *G. lucidum* and *V. volvacea* on six glycosidases

Enzyme tested	Inhibition (%) at 1 mg/ml of *G. lucidum*	Inhibition (%) at 10 mg/ml of *V. volvacea*
α-D-glucosidase (Brewers yeast)	70.0	0
ß-D-glucosidase (Almonds)	21.0	11.1
α-D-mannosidase (Jack beans)	< 10	25.5
ß-D-mannosidase (Snail acetone powder)	< 10	48.3
α-D-galactosidase (*E. coli*)	79.5	ND
ß-D-galactosidase (*A. oryzae*)	< 10	12.2

ND: not determined. No inhibition reported by Tsang, W and Y. S. Wong[56].

It was noted that crude extract of *G. lucidum* showed inhibitory effects on brewers yeast α-D-glucosidase and *E. coli* α-D-galactosidase. The inhibitory effect on ß-D-glucosidase was less. *V. volvacea* exhibited a various degree of inhibition on different glycosidase. The inhibition was less and non-specific as compared to *G. lucidum*.

IV.4.2 Inhibitory Effects of Partially Purified *G. lucidum* Extract on Glycosidase

The partially purified extract was obtained from dialysis of the crude extract (see Methods). It contain molecules with MW <2000. Figures IV.1 and IV.2 show that the inhibitory effects on glycosidases were concentration dependent, indicating that the inhibition is not due to nonspecific binding to enzymes resulting in inactivation.

Figures IV. 1 shows that up to 1 mg/ml, the partially purified extract exhibited over 80% inhibition on brewers yeast α-D-glucosidase. It was also found to have potent effect on *E. coli* α-D-galactosidase. The inhibition was about 83 % at 1 mg/ml of the extract (Fig. IV.2).

For almond ß-D-glucosidase, the extract had only slight effect on the enzyme, with 25 % inhibition at 1 mg/ml. (Fig. IV.3)

IC_{50} is defined as the concentration of an inhibitor exhibiting 50 % inhibition on the particular enzyme. The lower the IC_{50} value, the more potent the inhibitor is. The IC_{50} for brewers yeast α-D-glucosidase and *E. coli* α-D-galactosidase were found to be 0.68 mg/ml and 0.35 mg/ml respectively. As compared with the data list on Table IV.2, inhibition on the three glucosidases increased as the extract was purified by dialysis to remove higher MW impurities. Results suggest that inhibition was caused by low MW components (MW <2000) of the extract.

IV.4.3 Effect of Endogenous Substrates on Glycosidase Activities

In order to study the possible effect of endogenous substrates of the fungal extract on glycosidase activities, pre-incubation of extract with the enzyme was carried out prior to the addition of synthetic substrate (i.e. *p*-nitrophenyl glycoside). Results show that the inhibition was not affected. This indicates that the inhibition of glycosidase (using *p*-nitrophenyl glycoside as substrate) was not due to the presence of endogenous substrates.

Moreover, the addition of some natural substrates such as maltose, sucrose and methyl-α-D-glucopyranose were also found to have less than 10 % inhibition on brewers yeast α-D-glucosidase, no matter that all have α-glycosidic bond in the structure. The possibility of the natural substrate competing with the artificial substrate and causing the inhibition during enzyme catalysis was ruled out.

IV.4.4 Results of Liquid Column Chromatography

In liquid column chromatography of the partially purified *G. lucidum* extract, the inhibitory activities of various fractions collected from each step were shown. The active component (fractions 4-9) was mainly eluted by $CHCl_3$: MeOH (5:1 to 4:1, v/v) in the first chromatography (Fig. IV.4). Further separation was then carried out by second chromatography. The active components (fractions 43-54) was eluted by $CHCl_3$: MeOH (3:1 to 2:1, v/v) (Fig. IV.5). The percentage recovery of each purification step are summarised in the following table (Table IV.3)

Table IV.3 A summary of % recovery on purification of putative inhibitors from *Ganoderma lucidum* extract

	% recovery
Crude extract	100
Partially purified extract	59.1
Fractions collected from the first chromatography	4.01
Fractions collected from the second chromatography	1.29
Fractions collected from the third chromatography	0.314

IV.4.5 Structure Determination and Characterization of Purified Compounds

Two compounds with α-D-glucosidase inhibitory activities were purified from *G. lucidum*. Both were UV-inactive and charred in concentrated sulphuric acid, hence they were carbon-containing compounds without chromophores. The R_f values of the two compounds on Kieselgel $60F_{254}$ plate developed with $CHCl_3$: MeOH (1:1, v/v) were 0.33 and 0.35 (Fig. IV.6). In $CHCl_3$: MeOH: NH_4OH (45:40:15, v/v/v), the R_f value of these compounds, named compound A and B were 0.46 and 0.50 respectively (Fig. IV.7). Both compounds A and B were subjected to structure analysis by ^1H NMR.

The ^1H NMR spectrum for compound A was given in Fig. IV.8. It showed a doublet at 4.6 and 5.2 which are typical of the anomers of a monosaccharide. It also showed a group of peaks between 3.2 to 4.0 ppm. After comparison with reference standards, it was found that compound A was an equilibrium mixture of D-glucose containing 60 % of the ß-form and 40 % of the α-form. The free glucose usually favours the equatorial ß-form in solution during equilibrium, about 64 % in room temperature. Probably the factor is that the equatorial hydroxyl group is more easily solvated than the axial one. The EI-CI mass spectrum shown in Fig. IV.9 had a peak at 163 corresponding to the fragment M^+ - OH. This spectrum supported the conclusion that compound A was glucose which has a molecular weight of 180.

Mass spectrum gave a molecular ion at 153 of compound B corresponding to the formula $C_5H_{12}O_5$ (Fig. IV.10). Compound B was then subject to NMR analysis (Fig.IV.11) Three sugars with MW 152, namely adonitol, xylitol and arabitol were used as reference standards (Fig. IV.12-14)

Compound B was determined to be arabitol based on NMR spectrum comparison.

IV.4.6 Inhibitory Activities of Compounds A and B against Brewers yeast α-D-glucosidase

Compound A (12 mg/ml), identified as an equilibrium mixture of D-glucose, exhibited 78.5 % inhibition on brewers yeast α-D-glucosidase (250 mU/ml). The inhibition was higher than that of D(+)-glucose standard (53.3 %). This might be due to some impurities in compound A.

Compound B, identified as arabitol, also showed different inhibitory activity as compared to arabitol standard. Compound B, at concentration of 4.8 mg/ml, gave 44.3 % inhibition whereas arabitol standard exhibited 59.5 % inhibition at 4 mg/ml.

Determination the amount of reducing sugar by anthrone method found out the reducing sugar in crude extract and partially purified extract was 152.11 $\pm$ 0.027 μg/mg and 117.06 $\pm$ 0.0017 μg/mg respectively. The IC_{50} of the partially purified extract was 0.68 mg/ml (see IV.4.2), therefore the amount of reducing sugar in this partially purified extract was about 80 μg/ml. The concentration was not high enough to account for the observed 50 %

inhibition on α-D-glucosidase. It was speculated that reducing sugar (mainly glucose) together with other compound(s) or impurities demonstrating the inhibitory effect on α-glucosidase.

IV.5 Discussion

Ganoderma lucidum extract was shown to inhibit three glycosidases, namely brewers yeast α-D-glucosidase, *E.coli* α-D-galactosidase and almonds ß-D-glucosidase. Two α-D-glucosidase "inhibitors" were identified as glucose and arabitol. Glucose is one of the product of glucosidase reaction. It's inhibition on α-D-glucosidase is not unexpected. The literature survey revealed the monosaccharides found in *G. lucidum* are only some common sugars (glucose, xylose, galactose, arabinose and mannose), mannitol and α-α fucose[11,58]. This is the first report on the occurrence of arabitol in *G. lucidum*.

It is generally suggested that the inhibitor performs its action by adopting the right conformation and occupying the active site of enzyme. Arabitol is a five carbon sugar with the flexibility of its open chain structure, It may fit the active site of the enzyme and thus prevents the binding of substrate to the enzyme.

The potential uses of glycosidase inhibitors are for making as anti-viral and anti-tumor agents. All the purified compounds are valuable to test their anti-tumor abilities in some preliminary screening bioassay such as Potato Disc Bioassay[1,4,18,20].

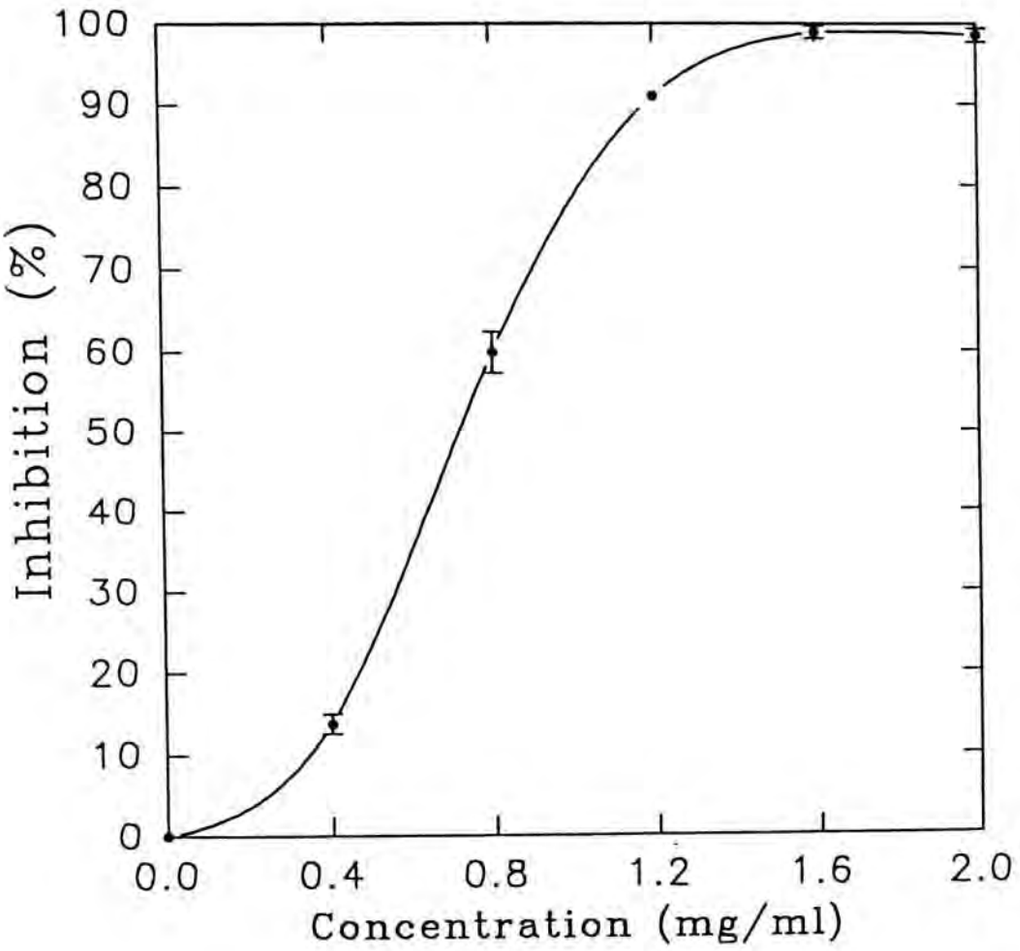


Fig. IV.1 Inhibition of brewers yeast α-D-glucosidase (250 mU/ml) by partially purified *G. lucidum* extract

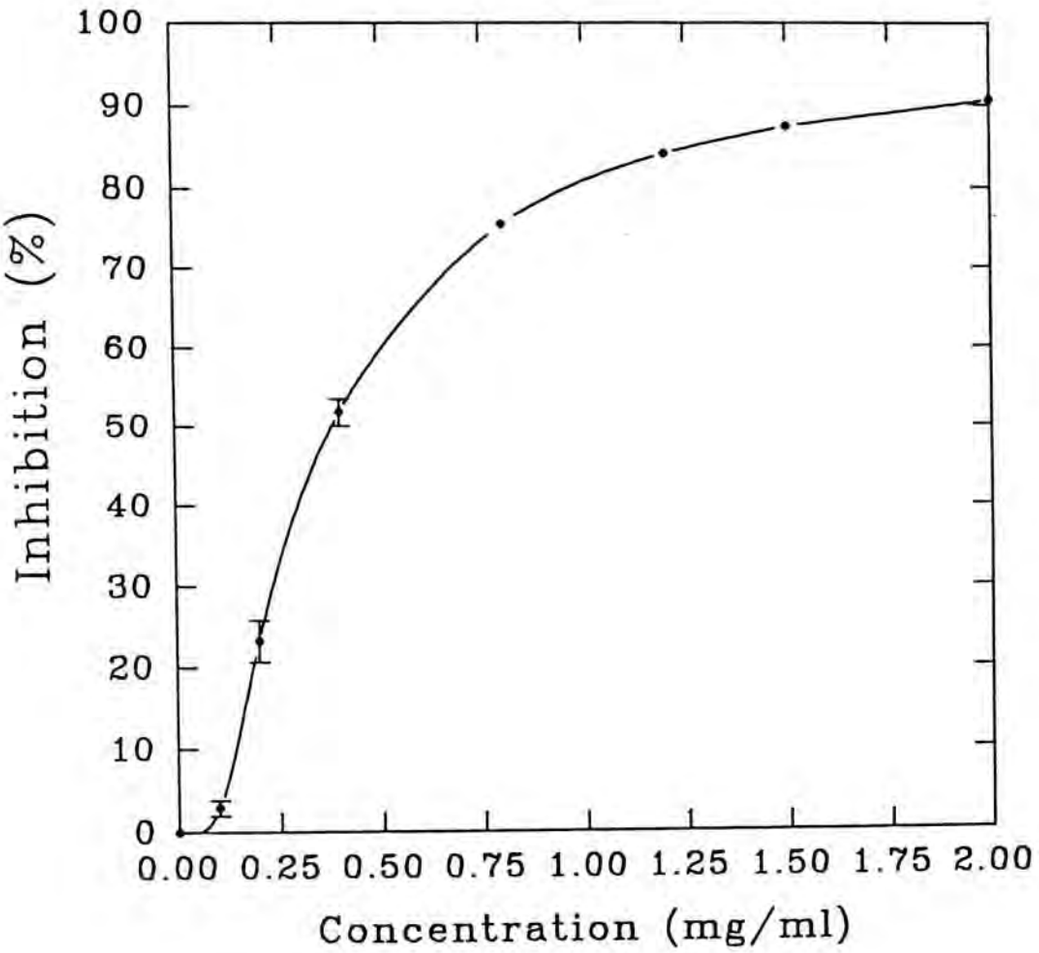


Fig. IV.2 Inhibition of *E. coli* α-D-galactosidase (50 mU/ml) by partially purified *G. lucidum* extract

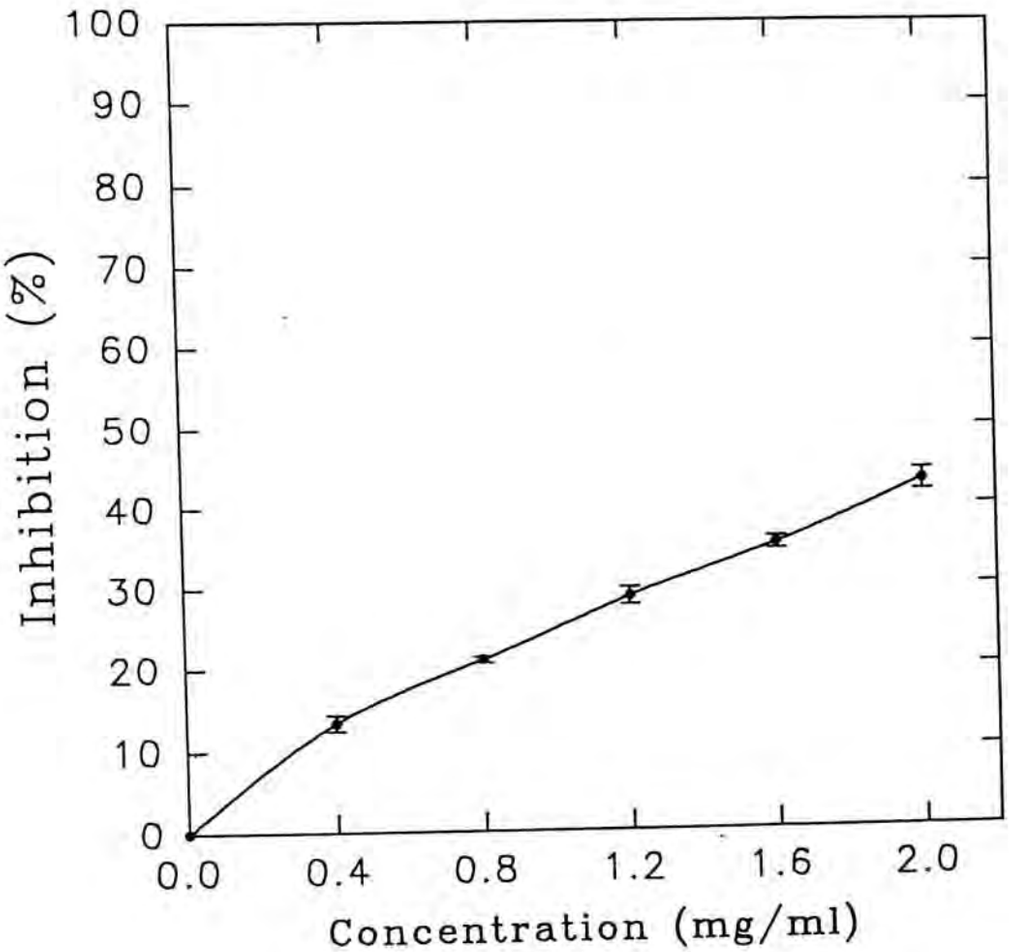
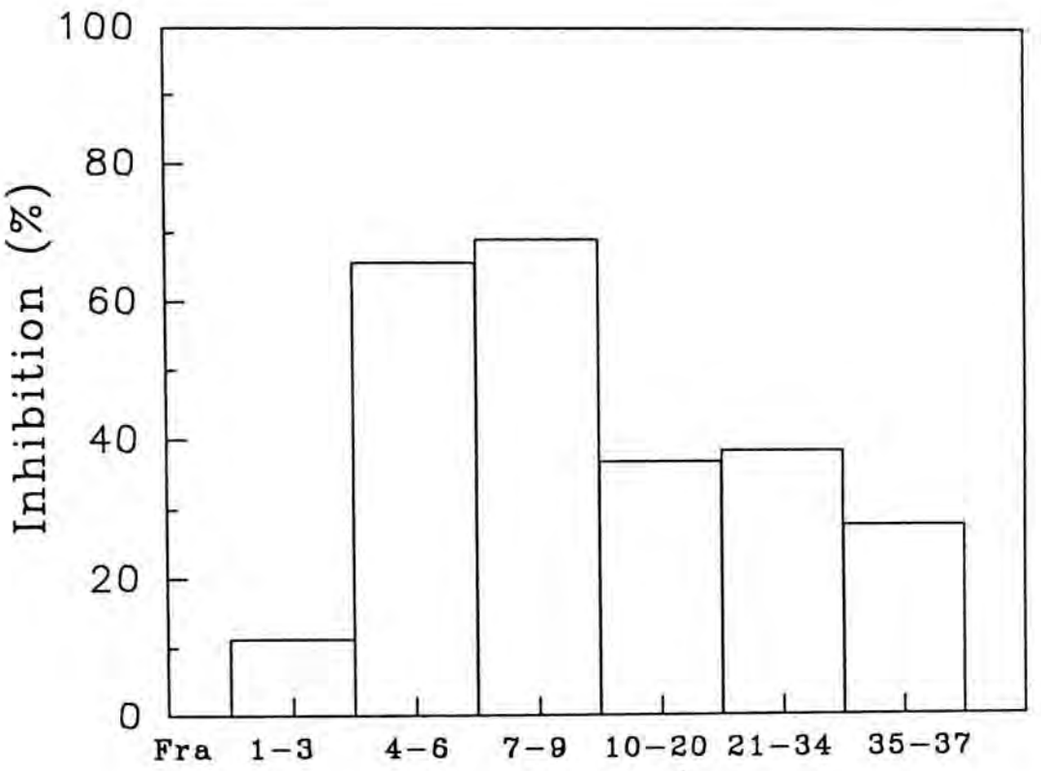
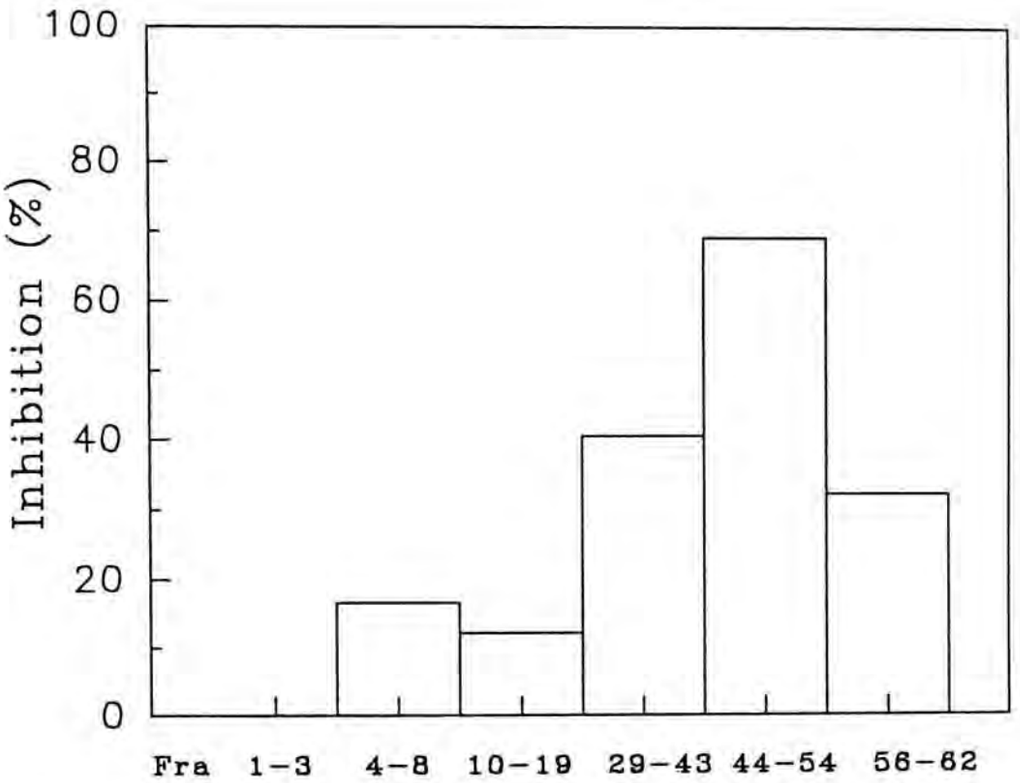


Fig. IV.3 Inhibition of almonds β-D-glucosidase (50 mU/ml) by partially purified *G. lucidum* extract



Fra 1-6 were eluted by $CHCl_3$: MeOH (5:1, v/v)
Fra 7-12 were eluted by $CHCl_3$: MeOH (4:1, v/v)
Fra 13-34 were eluted by $CHCl_3$: MeOH (3:1, v/v)
Fra 35-37 were eluted by $CHCl_3$: MeOH (1:1, v/v)

Fig.IV.4 Inhibition activities of various fractions collected from the first chromatography on brewers yeast α-glucosidase (250 mU/ml)



Fra 28-34 were eluted by $CHCl_3$: MeOH (8:1, v/v)
Fra 35-37 were eluted by $CHCl_3$: MeOH (6:1, v/v)
Fra 38-42 were eluted by $CHCl_3$: MeOH (5:1, v/v)
Fra 43-49 were eluted by $CHCl_3$: MeOH (3:1, v/v)
Fra 50-57 were eluted by $CHCl_3$: MeOH (2:1, v/v)
Fra 58-62 were eluted by $CHCl_3$: MeOH (1:1, v/v)

Fig. IV.5 Inhibition activities of various fractions collected from the second chromatography on brewers yeast α-glucosidase (250 mU/ml)

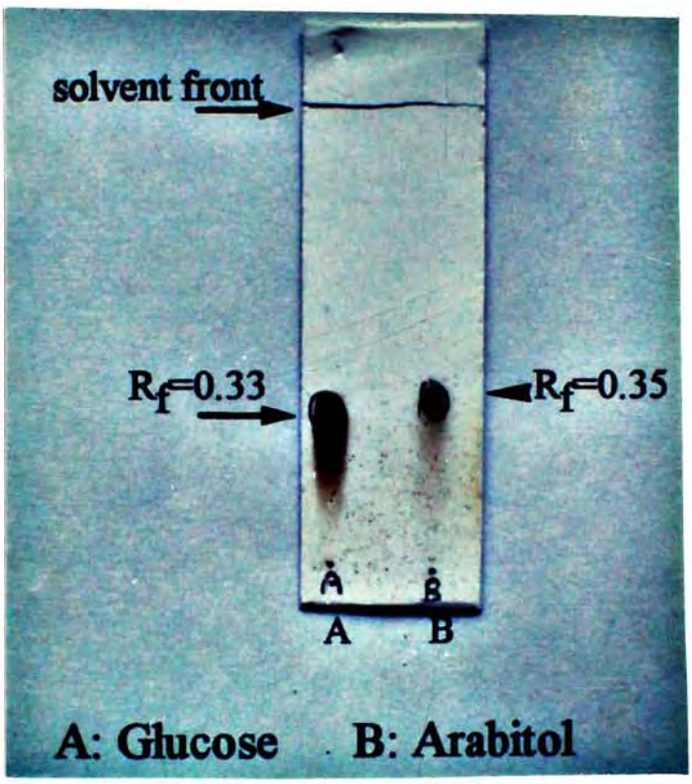


Fig. IV.6 Chromatogram of compound A and B in the developing solvent $CHCl_3$: MeOH (1:1, v/v)

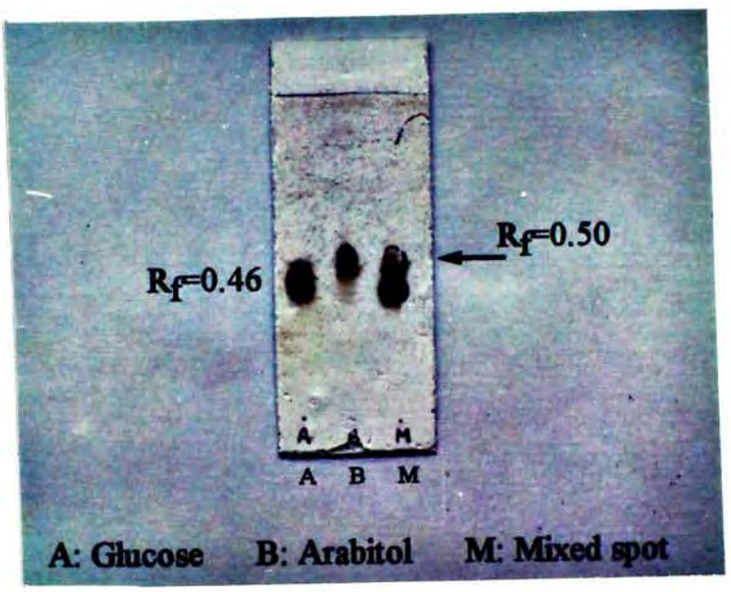


Fig. IV.7 Chromatogram of compound A and B in the developing solvent $CHCl_3$: MeOH: NH_4OH (45:40:15, v/v/v)

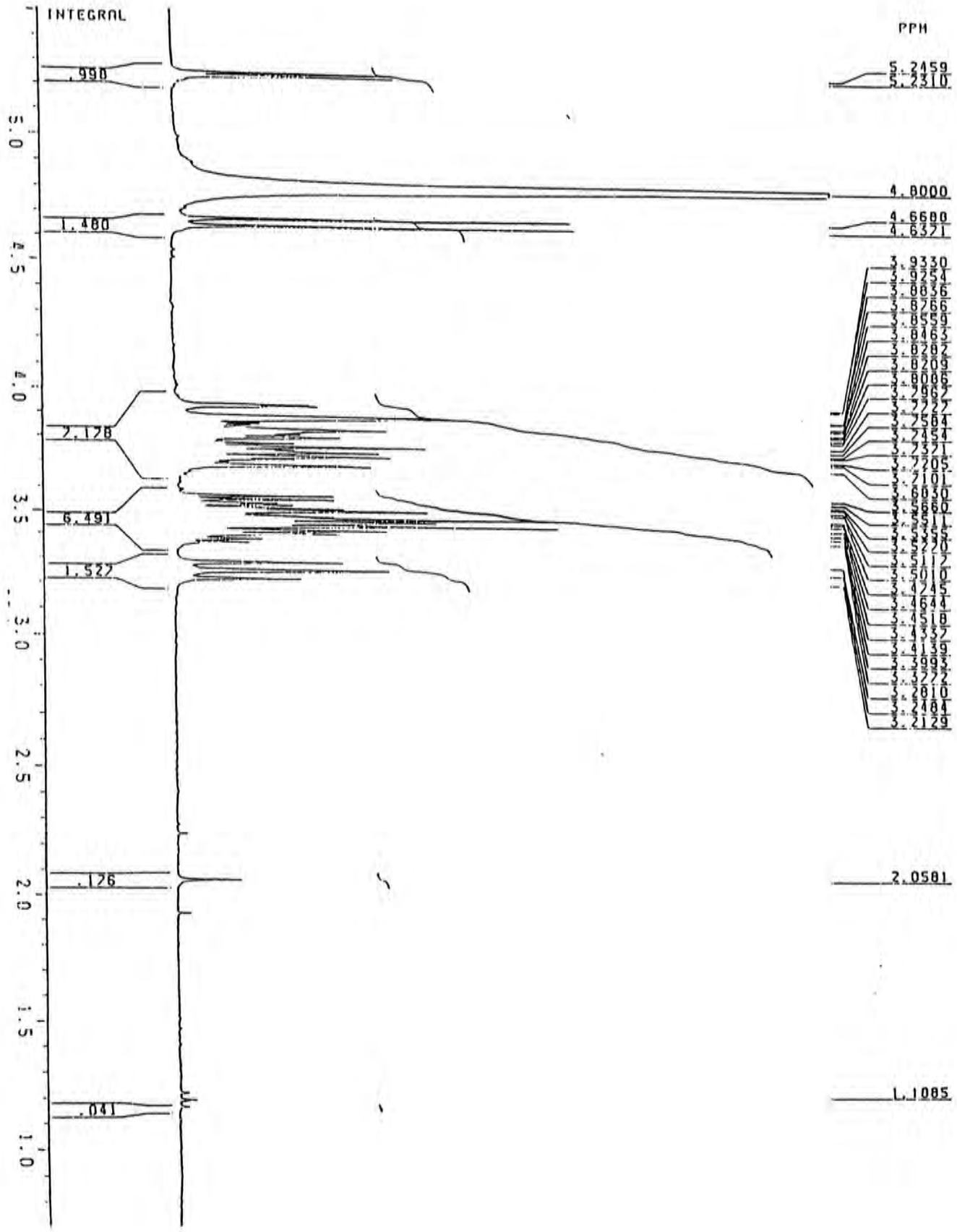


Fig. IV.8 NMR spectrum of compound A in D_2O solvent

Fig. IV.9 EI-CI mass spectrum of compound A

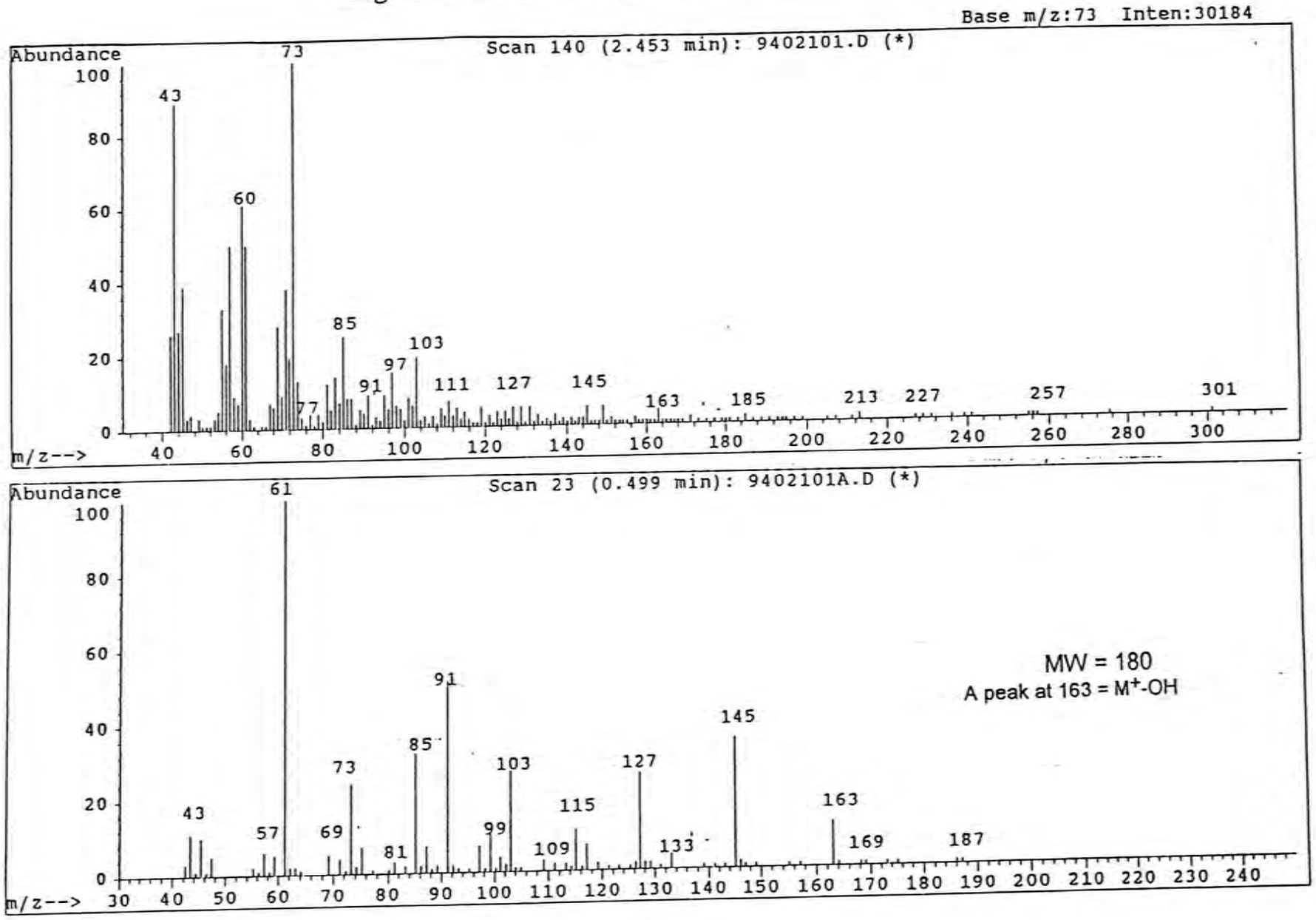


Fig. IV. 10 EI-CI mass spectrum of compound B

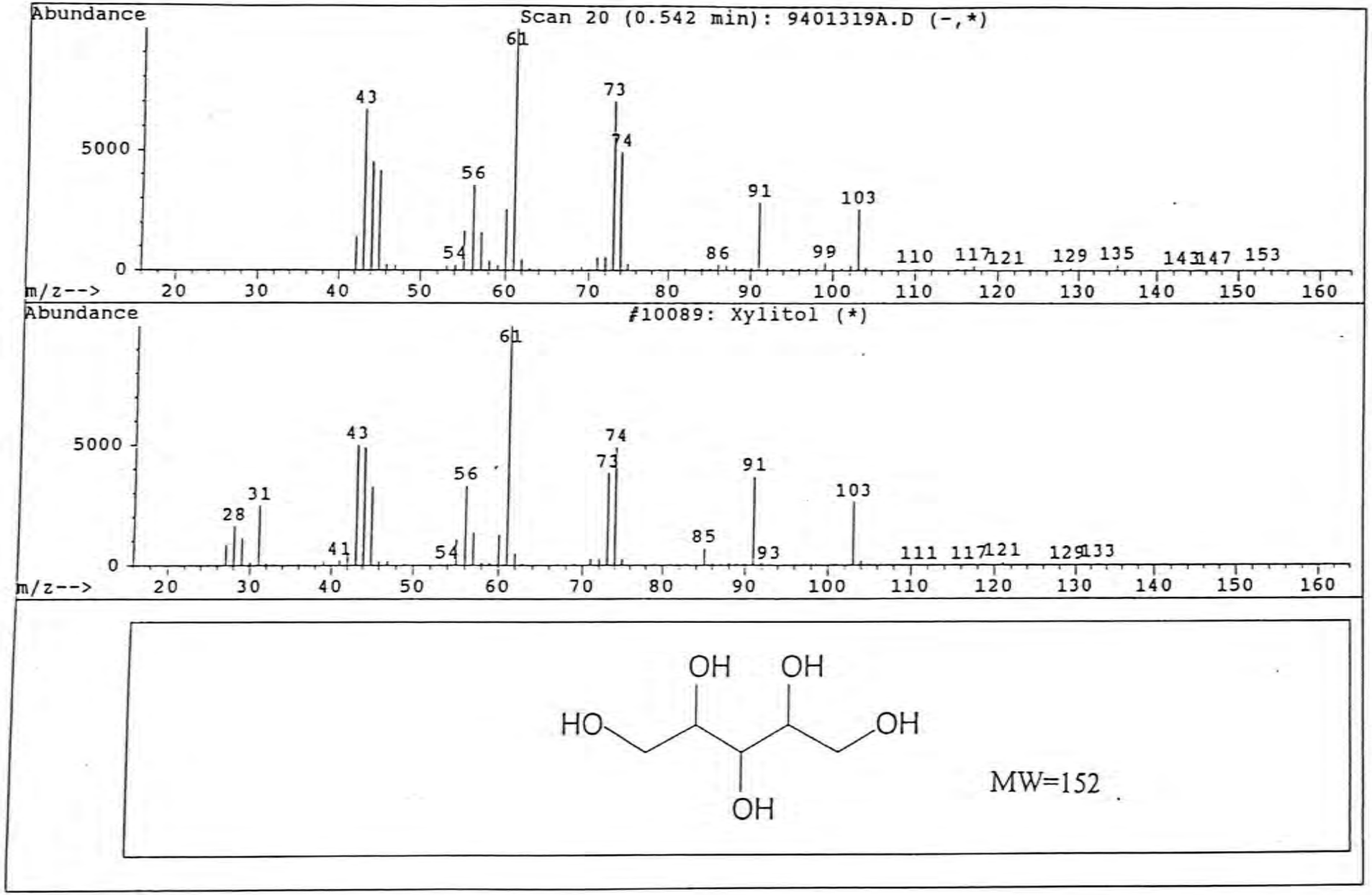


Fig. IV. 11 NMR spectrum of compound B in D_2O solvent

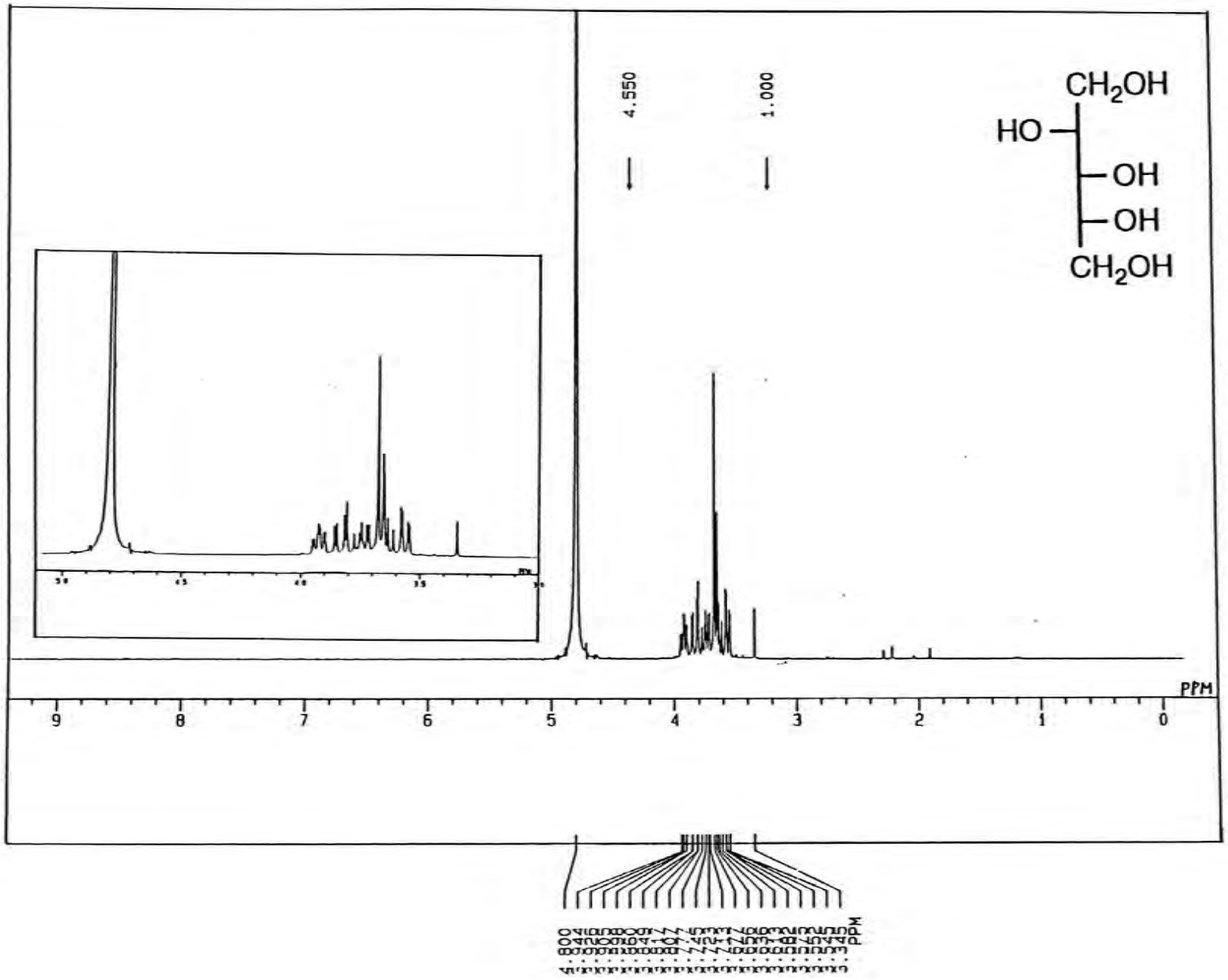


Fig. IV.12 NMR spectrum of adonitol in D_2O solvent

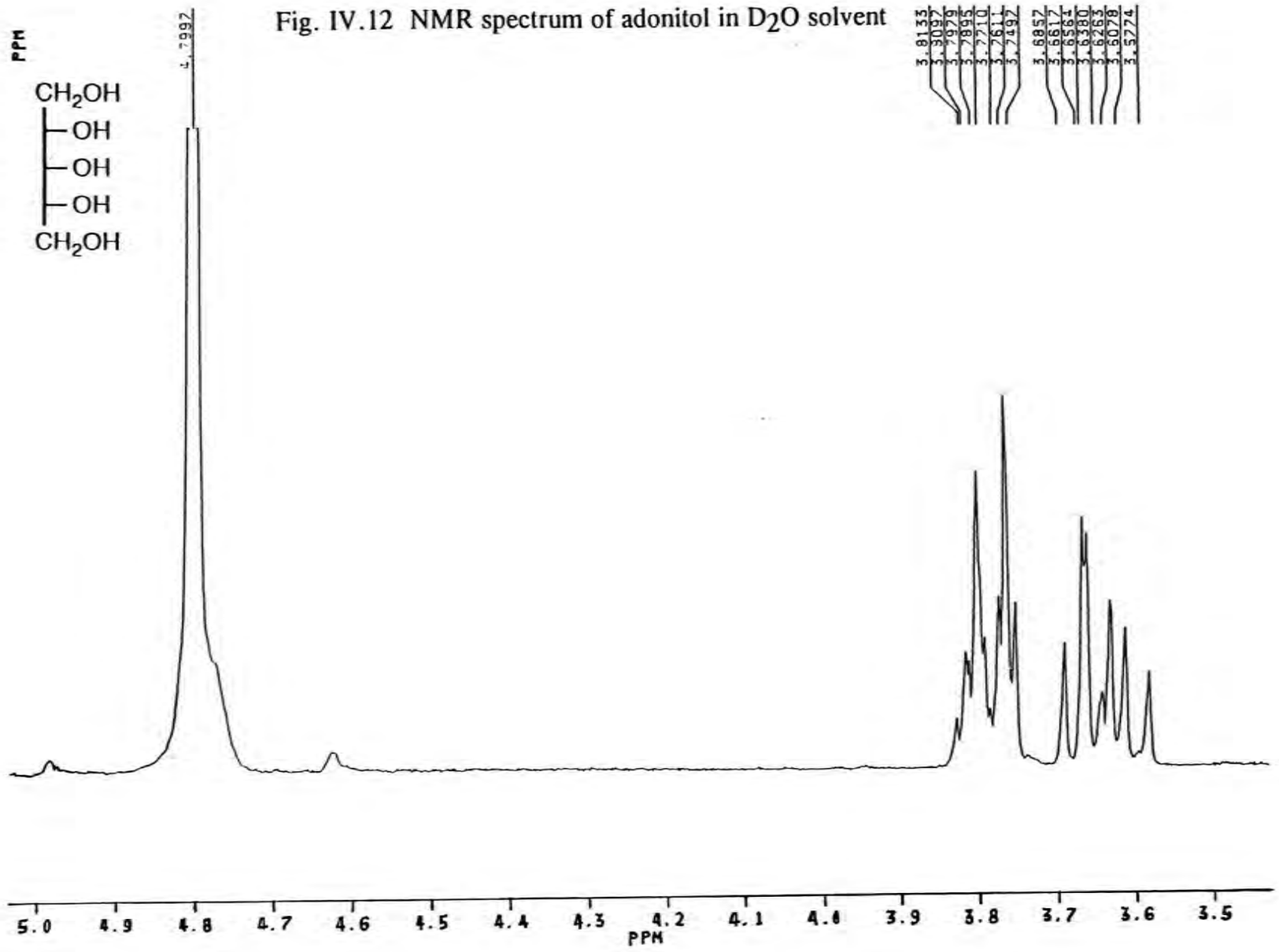


Fig. IV.13 NMR spectrum of xylitol in D_2O solvent

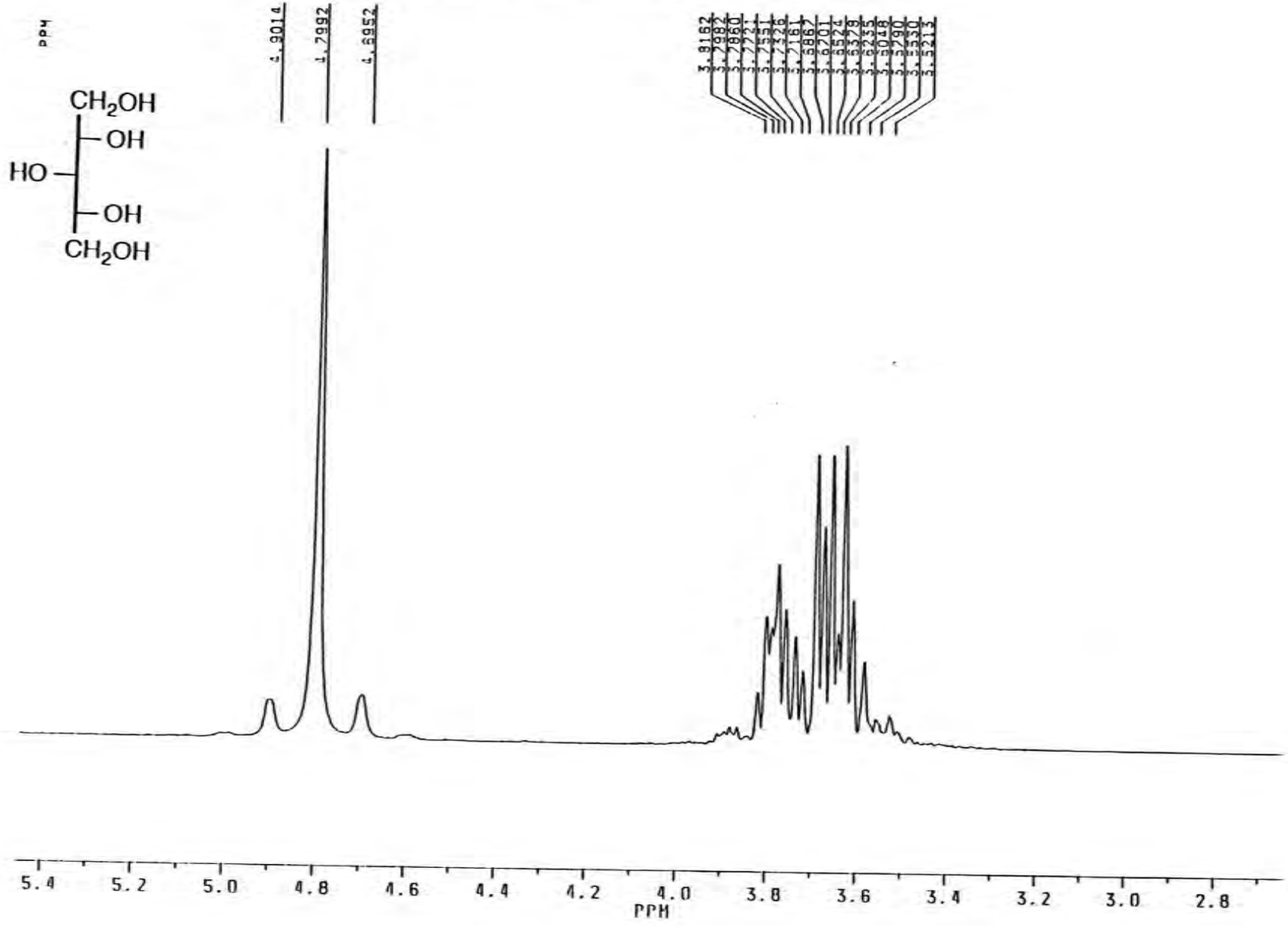
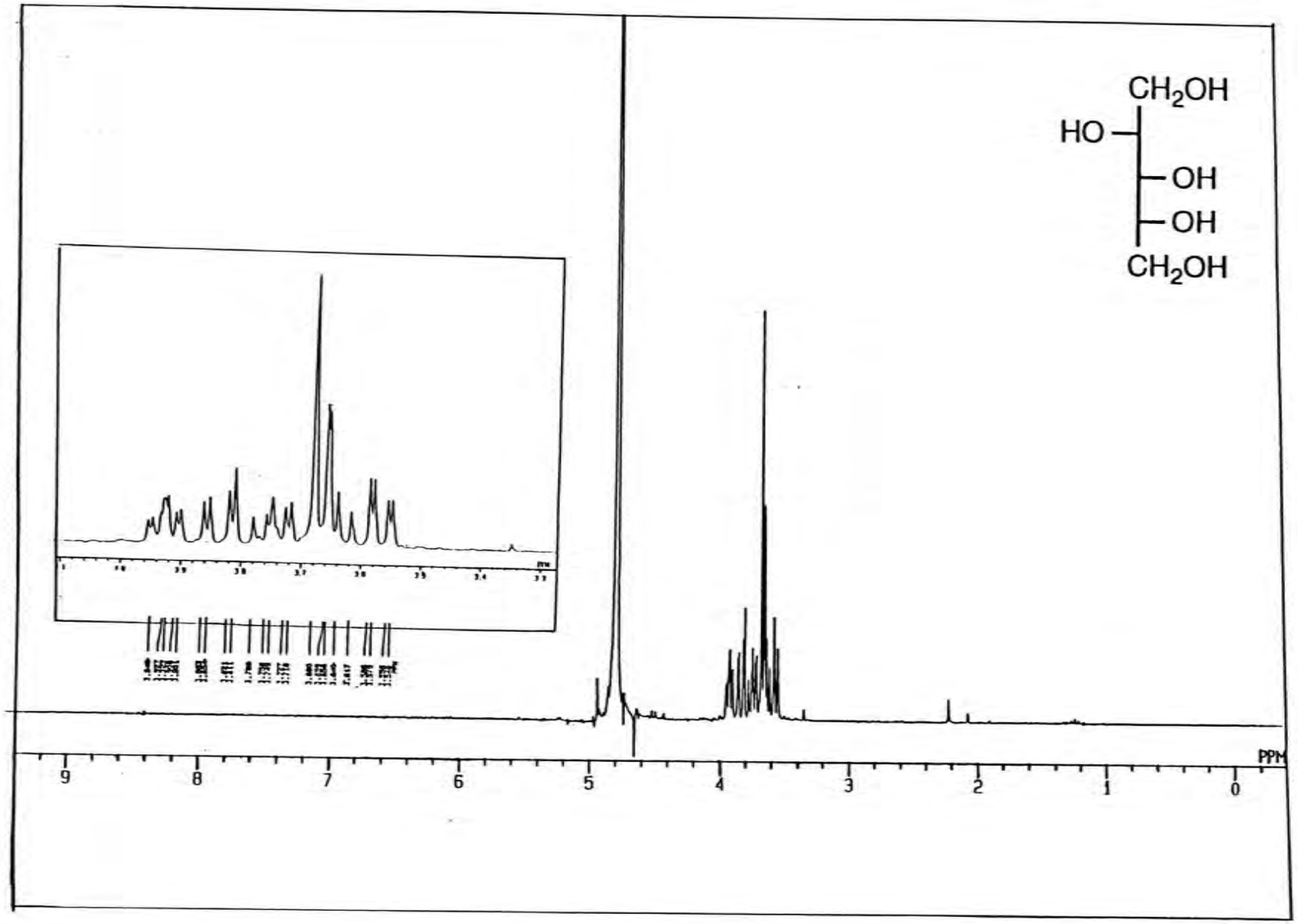


Fig. IV.14 NMR spectrum of arabitol in D_2O solvent



Chapter V Conclusion

Glycosidases are enzymes responsible for glycoprotein processing. Inhibition of these enzymes has implication for both anti-viral and anti-tumor chemotherapy. Plant alkaloids are well known glycosidase inhibitors which have been confirmed to display anti-virus, anti-tumor and anti-AIDs activities. However, the toxicity limited the uses of these compounds[38]. Exploitation of the potent glycosidase inhibitors with less toxicity is currently under extensive study.

Two approaches are currently undertaken to develop glycosidase inhibitors as antiviral agents. One is to identify and isolate novel naturally occurring glycosidase inhibitors from various sources, including microorganisms and plants. Another approach is to synthesize and modify existing potent glycosidase inhibitors.

In this study, naturally occurring glycosidase inhibitors are being searched from different fungi. Since *Ganoderma lucidum* belongs to the same family as *Phellinus* which has been reported to produce a potent glycosidase inhibitor, cyclophellitol, this mushroom was chosen for detailed study. By a series of chromatographic separation, two compounds with α-D-glucosidase inhibitory activities were isolated. They were identified as the equilibrium

mixture of D-glucose and arabitol. *G. lucidum* extract also exhibited inhibitory activity on *E. coli* α-D-galactosidase; the identification of putative α-D-galactosidase inhibitor(s) will be the objective of future study.

Two classes of synthetic glycosidase inhibitors were prepared by Chemistry department. One is cyclophellitol and its analogues which belong to the class of irreversible inhibitor. Each compound was found to inhibit the configurationally related glycosidase. It is suggested that the probable inactivation mechanism is due to the opening of epoxide ring at C-1 and then forming covalent bond with amino acid residues at the active site. As concluded, the inhibitors are glycosidase-specific with respect to the stereochemistry of epoxide ring at C-1. Shifting the epoxide would affect the close proximity to the enzyme and result in low inhibitory power.

The other type is aminocyclitols which was proved to be the reversible competitive inhibitor. The flattened, flexible conformation and a positive charge in cyclic structure made them as another kind of glycosidase inhibitors. However, the weak basic properties of these compound may greatly affect their inhibition specificity. In addition, the inhibition potencies are lower compared with the cyclophellitol-like compounds mentioned before. By means of structure-function studies of above synthetic compounds, the important requirements for sugar analogues to be glycosidase inhibitors could be

revealed.

Some of synthetic compounds are novel compounds and found to have specific inhibition on glycosidase. These compounds which are devoid of toxicity and show a narrow specificity may be of interest for physiological studies and clinical use.

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